The role of nutrients in G_1 phase progression of *Saccharomyces cerevisiae*

De rol van nutrienten in progressie door de G_1 fase van

Saccharomyces cerevisiae

(met een samenvatting in het Nederlands)

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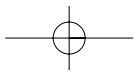
Rare jongens,

die wetenschappers

Obelix, galliër



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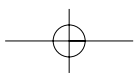

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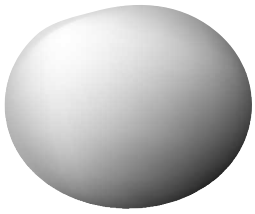
List of abbreviations:

CAK:	cyclin-dependent kinase activating kinase
CDC:	cell division cycle
CDK:	cyclin-dependent kinase
CKI:	cyclin-dependent kinase inhibitor
DAG:	diacylglycerol
DW:	dry weight
HA:	hemagglutinin
HOG:	high osmolarity growth
MAP:	mitogen-activated protein
MAPK:	mitogen-activated protein kinase
MAPKK:	mitogen-activated protein kinase kinase
MAPKKK:	mitogen-activated protein kinase kinase kinase
MBF:	Mlu1/Swi6-dependent cell cycle box binding factor
MCB:	Mlu1/Swi6-dependent cell cycle box
MEK:	mitogen-activated protein kinase kinase
MEKK:	mitogen-activated protein kinase kinase kinase
PEST:	region rich in proline (P), glutamine (E), serine (S) and threonine (T)
PI:	phosphatidylinositol
PKA:	protein kinase A
PKC:	protein kinase C
PLC:	phospholipase C
SBF:	Swi4/Swi6-dependent cell cycle box binding factor
SCB:	Swi4/Swi6-dependent cell cycle box
STRE:	stress-responsive element
TOR:	target of rapamycin

Chapter 1

General Introduction.



Introduction

Organisms that have optimally integrated fast reproduction with high survival have a strategic advantage over less adapted species during evolution. However, during their reproductive stage they are more susceptible to environmental stresses. This thesis focuses on the main decision point in the life cycle of the yeast *Saccharomyces cerevisiae*, in which the cell decides to rapidly grow and divide, or accumulate intracellular carbohydrates and delay cell cycle progression.

The cell cycle of *Saccharomyces cerevisiae*

Growth and division of eukaryotic cells are well-coordinated processes. During the cell division cycle, all essential components are duplicated and the cell mass is doubled. The process of cell division is tightly controlled in the mitotic cell cycle, in which the different steps of the duplication process are separated in time (Alberts, 1994).

The cell cycle is a continuous process that can be divided into four successive phases called the G_1 phase, S phase, G_2 phase and M phase (figure 1). As a new cell is formed, it remains in the G_1 phase until conditions are favourable for completion of the total cell cycle. When all requirements are met, the cell enters the S-phase and DNA-replication takes place. Subsequently, the cell prepares for mitosis in the G_2 phase and enters the M phase, in which mitosis and cytokinesis are performed.

The cell cycle of the yeast *Saccharomyces cerevisiae* is characterised by the formation of a bud as the cell enters the S phase. During the rest of the cell cycle, this bud will grow until a viable daughter cell has formed that is separated from the mother cell at the end of the M phase. In contrast to other eukaryotic cells, division of *S.cerevisiae* is an asymmetric process in which the newly formed cell is smaller than the mother cell (Hartwell & Unger, 1977). After division, the daughter cell has to grow in size before it can initiate a new cell cycle, while the mother cell can enter the S phase almost immediately.

As a first step in bud formation, the cell determines the location of the site of budding at the end of the G_1 phase. The actin cytoskeleton, which is normally distributed randomly over the cell in actin cables and patches, is reorganised and polarised at the "pre-bud" site (figure 1; Adams & Pringle, 1984; Kilmartin & Adams, 1984). Actin cables and microtubules are oriented towards the budding site, while various proteins involved in morphogenesis and secretion accumulate at the polarised tip of the cell (Drubin & Nelson, 1996; TerBush & Novick, 1995). One group of proteins,

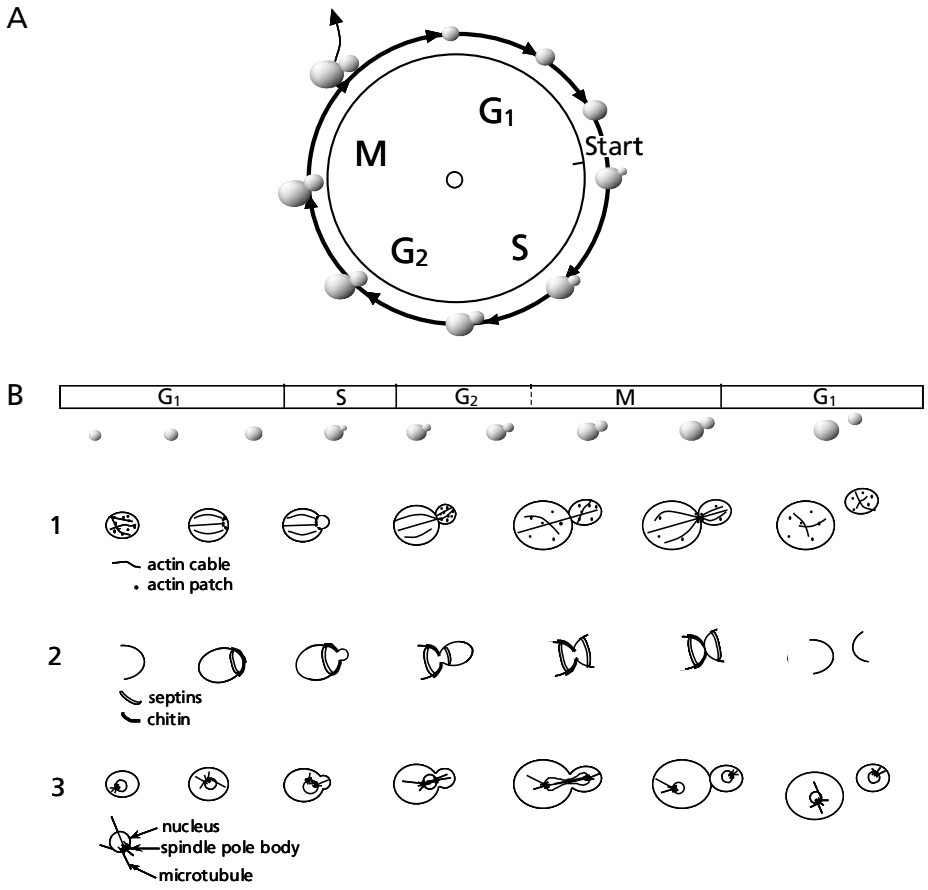


Figure 1: **The cell cycle of *S. cerevisiae.*** *A*. The cell cycle consists of 4 phases: the G_1, S, G_2 and M phase. During the G_1 phase the cell increases in size until the cell starts budding at 'Start'. After DNA is duplicated in the M phase, the cell prepares for mitosis in the G_2 phase and enters the mitotic M phase. *B*. The major events during the yeast cell cycle. *1*. Rearrangement of the actin cytoskeleton: actin patches are directed to the site of budding in late G_1 and late M phase. Actin cables direct the secretion machinery towards the bud from late G_1 until the end of the G_2 phase. *2*. Septin formation and chitin deposition. Septin filaments arrange at the plasmamembrane at the site of budding in late G_1 and chitin is deposited in the overlying cell wall. In late M phase, a septum is formed between mother and daughter cell. *3*. Microtubuli and spindle pole body (SPB) localization. The SPB's are oppositely positioned in the G_2 phase and the nucleus is moved towards the budding site via the microtubule. In the M phase, the DNA is segregated by action of the microtubuli.

the septins, assemble in a ring at the pre-bud site and form filaments at the budding site (Longtine *et al*., 1996). Chitin synthases deposit a ring of chitin in the overlying cell wall as a primary septum between mother and daughter cell (Bulawa, 1993; Shaw *et al*., 1991). As the bud is formed, the nucleus migrates towards the budding site via microtubules (Copeland & Snyder, 1993; Davis & Fink, 1990; Jacobs *et al*., 1988; Sullivan & Huffaker, 1992). During growth of the bud, the nucleus is extended into the bud via the mother-bud neck and the duplicated DNA is equally divided over mother and bud (Davis & Fink, 1990). During cytokinesis a secondary, nonchitinous septum is formed on either side of the first septum (Shaw *et al*., 1991). After degradation of the first septum by chitinase, the cells are separated and the cytoskeleton is depolarised (Kilmartin & Adams, 1984; Kuranda & Robbins, 1991).

Start; the point of no return

At the end of the G_1 phase, the decision is made to initiate bud formation and progress through the cell cycle. After passing this "point of no return", the cell is committed to finish the total cell cycle until the next G_1 phase. This decision point is also known as "Start" (Pringle, 1981).

The decision to pass Start is dependent on the environmental conditions. Early in the G_1 phase, cells are able to enter a number of developmental stages. When nutrients are abundant, cells will generally pass Start and proceed with the cell cycle. Under nutrient-limitation, cells accumulate reserve carbohydrates and reside in a quiescent state in the G_1 phase or begin sporulation (Kupiec, 1997; Pringle, 1981). Exposure to pheromone of the opposite mating type also results in an arrest in G_1, followed by initiation of mating (figure 2; Marsh, 1997). The decision to enter the correct developmental program in the G_1 phase is crucial for survival of the cell, e.g. nutrient-deprived cells become inviable if they continue to proceed through the cell cycle and well-fed cells initiating sporulation will be overgrown by dividing cells. Therefore, the cell must monitor the environment continuously and adapt the cell cycle to external conditions.

The signalling between the cell and the environment is regulated by a multitude of signal transduction cascades. One signal transduction family, the mitogen-activated protein kinase (MAPK) pathway family, consists of different protein kinase cascades that can be activated by environmental changes (Gustin *et al*., 1998). Prior to passage of Start in G_1, several MAPK pathways can be activated and affect the decision to progress through the cell cycle (see paragraph MAPK pathways).

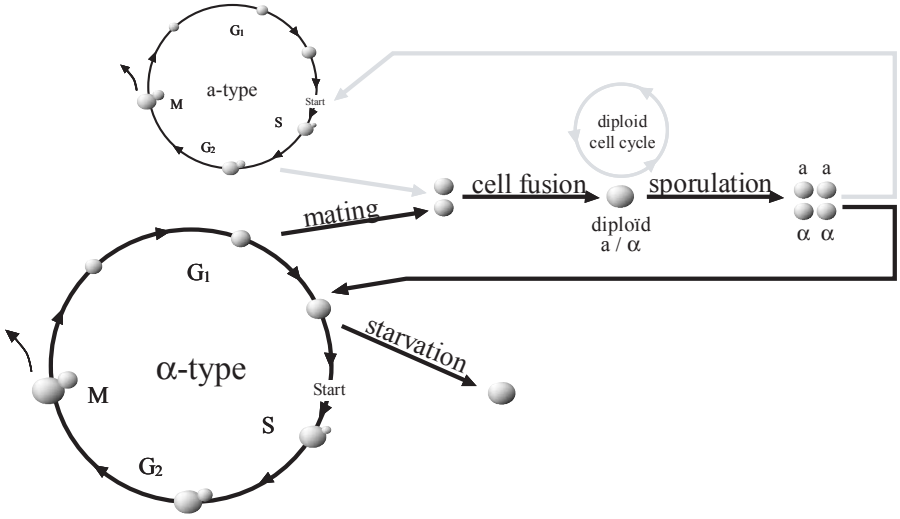


Figure 2: **The lifecycle of *S.cerevisiae***. The main choices in the lifecycle are made during the G_1 phase, when the cell decides to enter a new division round, remain in the G_1 phase upon starvation or mate with a cell from the opposite mating type. Upon mating, haploid cells fuse to form a diploid cell, which can either sporulate or enter the cell cycle as a diploid cell.

The cell cycle at the molecular level

The main regulators of the cell cycle of *S. cerevisiae* and other eukaryotes are the cyclin-dependent kinases (CDKs) (Lees, 1995; Nigg, 1995). CDKs are serine/ threonine kinases that can be activated throughout the cell cycle by binding of different cyclins. One of the five CDKs in *S. cerevisiae*, Cdc28, controls the major events of the yeast cell division cycle (Mendenhall & Hodge, 1998).

The cyclin-dependent kinase Cdc28

The activity of Cdc28 depends on several activating and inhibitory proteins. Activation of Cdc28 requires phosphorylation of threonine 169 of the protein and the binding of a cyclin (Deshaies & Kirschner, 1995). The phosphorylation step on T169 is performed by activity of Cak1, a CDK-activating kinase (CAK) (Espinoza *et al*., 1996; Kaldis *et al*., 1996; Thuret *et al*., 1996). The Cak1 protein is constitutively active during the cell cycle, resulting in continuously phosphorylated T169 of Cdc28 (Amon *et al*., 1992; Espinoza *et al*., 1996; Sutton & Freiman, 1997). Therefore, the main regulation of activating Cdc28 activity through the cell cycle is not controlled by phosphorylation on

T169, but rather by temporal binding of the different cyclins (see next paragraph).

Cdc28 activity is also regulated by binding to a CDK inhibitor (CKI) and by inhibitory phosphorylation on tyrosine 19 or threonine 18 (Mendenhall & Hodge, 1998). Whereas the regulators of T18 phosphorylation remain to be identified, Y19 can be phosphorylated by protein kinase Swe1 and dephosphorylated by protein phosphatase Mih1 (Booher *et al*., 1993; Russell *et al*., 1989). During the cell cycle, phosphorylation on Y19 does not affect Cdc28 activity during the G_1 phase (Booher *et al*., 1993). The CKI proteins Far1 and Sic1 can bind to Cdc28 and exclude cyclins from the Cdc28 active site (Mendenhall & Hodge, 1998). Both Far1 and Sic1 are expressed during the G_1 phase and are rapidly degraded as cells pass Start. By binding of Far1 or Sic1 to different cyclin-Cdc28 complexes in the G_1 phase, cyclin-specific Cdc28 activity is impaired and cells remain in the G_1 phase (Donovan *et al*., 1994; McKinney *et al*., 1993; Schwob *et al*., 1994). The Far1 protein can only inhibit Cdc28-cyclin activity upon phosphorylation by the pheromone response pathway (see paragraph MAPK pathways). The Sic1 protein inhibits the activity of Cdc28 bound to B-type cyclins during the G_1 phase, as described in the next paragraph. Unlike Far1, no additional activation step is required for inhibition of Cdc28 activity by Sic1. Therefore, the inhibitory action of the Sic1 protein is only regulated by degradation by the proteasome.

The cyclins

Cyclins are unstable proteins that are periodically expressed throughout the cell cycle (figure 3). After a cyclin becomes expressed, the protein binds and activates a CDK and subsequently becomes rapidly degraded by the proteasome. During the cell cycle, different cyclins are temporarily expressed and regulate cell cycle progression by activation and inhibition of specific cell cycle stages (Lew, 1997).

The Cdc28-binding cyclins can be classified into two groups: the G_1-specific cyclins and the B-type cyclins. The G_1-specific cyclins Cln1, Cln2 and Cln3 activate Cdc28 during the G_1 phase, whereas the activity of the six B-type cyclins, Clb1-6, is subdivided over the other phases of the cell cycle (figure 3).

At the beginning of the G_1 phase, cyclin Cln3 is the first cyclin to activate Cdc28 (Dirick *et al*., 1995; Stuart & Wittenberg, 1995). Although Cln3 is expressed throughout the cell cycle (Cross & Blake, 1993; Tyers *et al*., 1993), it only activates Cdc28 during the G_1 phase, when no other cyclins are present. The Cdc28 / Cln3 complex activates transcription of both the late G_1 cyclins Cln1 and Cln2 and the B-type cyclins Clb5 and Clb6 at the end of the G_1 phase (Epstein & Cross, 1992; Kuhne & Linder, 1993; Schwob & Nasmyth, 1993). The Clb5, 6 / Cdc28 complex remains inactive during the G_1 phase, as Clb / Cdc28 activity is inhibited by the CKI Sic1 (Mendenhall, 1993; Schwob *et al*., 1994).

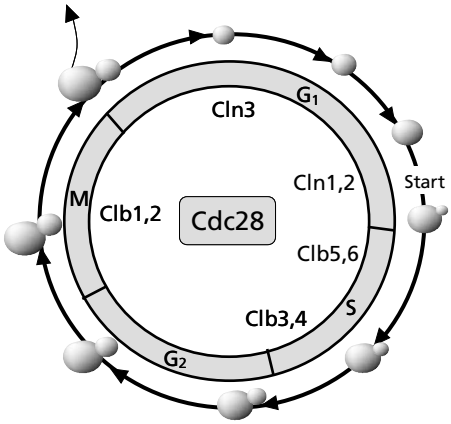


Figure 3: **The cell cycle of *S.cerevisiae* at the molecular level.** During the cell cycle, the different cyclins bind and activate the main cyclin-dependent kinase Cdc28. All cyclins but *CLN3* are temporarily expressed during the cell cycle and the period at which the cyclin activates Cdc28 is shown.

Upon activation of Cdc28 by binding of Cln1 and Cln2, Sic1 is degraded by the proteasome and cells pass Start (Schneider *et al*., 1996; Verma *et al*., 1997). Together with Sic1 the G_1 cyclins are degraded (Lanker *et al*., 1996; Willems *et al*., 1996), leaving Clb5 and Clb6 to activate Cdc28 and trigger the cell to enter the S phase (Schwob *et al*., 1994). The Clb5, 6 / Cdc28 activity initiates DNA replication and prevents reinitiation of DNA replication origins that have already 'fired' (Dahmann *et al*., 1995). In mid-S-phase Clb5 and Clb6 are degraded and Clb3 and Clb4 become expressed until anaphase (Fitch *et al*., 1992; Richardson *et al*., 1992; Surana *et al*., 1991). It is believed that Clb3/Clb4-induced Cdc28 activity plays a role in spindle pole formation, though Clb1 and Clb2 are also able to fulfil this function (Amon *et al*., 1993; Richardson *et al*., 1992). Clb1 and Clb2 are expressed just prior to the anaphase and promote spindle elongation (Richardson *et al*., 1992). Furthermore, the Clb1, 2 / Cdc28 activity negatively regulates expression of several G_1 specific genes and inhibits bud formation by depolarising the cytoskeleton (Amon *et al*., 1994; Koch *et al*., 1996).

The different cyclins have many overlapping functions and not all cyclins are required for progression through the cell cycle. Deletion studies have shown that cells deleted for all three G_1 cyclins arrest in the G_1 phase, but the activity of either G_1 cyclin overcomes this growth defect (Cross, 1990; Richardson *et al*., 1989). Nevertheless, cells lacking one or two G_1 cyclins show elongation of the G_1 phase duration (Dirick *et al*., 1995; Stuart & Wittenberg, 1995). The overlapping and additional function of the

different cyclins is also reflected in the localisation of the G_1 cyclins: Cln3 is localised predominantly in the nucleus, whereas Cln1 and Cln2 are cytoplasmic proteins (Miller & Cross, 2000). The B-type cyclins also show a distribution of overlapping functions between the different cyclins pairs, but their functional complementation seems to be more complex (Mendenhall & Hodge, 1998).

Regulation of progression through the G_1 phase

The main components regulating progression through the G_1 phase are the CDK Cdc28, the G_1 cyclins Cln1, Cln2 and Cln3 and the transcription factors Swi4 and Swi6. During the G_1 phase, activation of Cdc28 by Cln3 induces transcription of Swi4 (Tyers & Futcher, 1993). Subsequently, transcription factor Swi4 can bind to Swi6 to form an inactive complex (Andrews & Moore, 1992; Sidorova & Breeden, 1993). Upon activation of this transcription complex by the MAP kinase Slt2 (Mpk1), the cyclins Cln1 and Cln2 become expressed (Madden *et al*., 1997). The Cln1, 2 / Cdc28 kinase activity then triggers the cell to enter the S phase (figure 4).

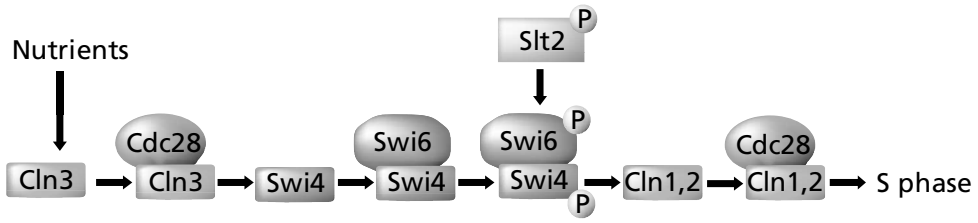


Figure 4: **A model of regulation of progression through the G_1 phase in *S.cerevisiae*.** Upon addition of nutrients, expression of CLN3 is induced in the beginning of the G_1 phase. Upon binding of Cln3p to the CDK Cdc28, SWI4 is expressed and binds to transcription factor Swi6. The Swi4/Swi6 (SBF) transcription complex is activated upon phosphorylation by the MAP kinase Slt2, which results in the transcription of CLN1 and CLN2. The late-G_1 cyclins Cln1p and Cln2p bind to Cdc28 and induce entry of the S phase.

The cyclin Cln3

The first step in G_1 phase progression at the molecular level is the increase of the amount of Cln3 in the cell. The Cln3 amount is regulated at the level of transcription and translation, as well as on the stability of the protein (Barbet *et al*., 1996; Belli *et al*., 2001; Hall *et al*., 1998; Polymenis & Schmidt, 1997).

At the transcriptional level, the amount of *CLN3* is dependent on the nutrient availability in the medium (Hubler *et al*., 1993; Parviz *et al*., 1998; Parviz & Heideman, 1998). Addition of a nitrogen or carbon source to nutrient starved cells increases the level of *CLN3* mRNA (Parviz & Heideman, 1998). Upon glucose addition, repeated sequences of A_2GA_5 in the promoter of *CLN3* are responsible for induction of the *CLN3* mRNA level in the cell (Parviz *et al*., 1998). Thus, increasing amounts of nutrients induce transcription of *CLN3*.

The translation of *CLN3* is regulated by phosphatidylinositol kinase homologues TOR1 and TOR2 (Barbet *et al*., 1996). Loss of the function of both TOR-genes (TOR= target of rapamycin) causes early inhibition of translation initiation and arrest of cells in the G_1 phase (Berset *et al*., 1998; Helliwell *et al*., 1998; Zaragoza *et al*., 1998). Alteration of translational control of *CLN3,* by fusing the *CLN3* open reading frame to the untranslated reader and promoter of the *UBI4* gene, suppresses this rapamycin-induced arrest in G_1 (Barbet *et al*., 1996).

The stability of the Cln3 protein is dependent on the carboxy-terminal region of the protein (Cross & Blake, 1993). This region contains PEST-sequences (rich in proline [P], glutamate [E], serine [S] and threonine [T]), which are phosphorylated and ubiquitinated, followed by degradation by the proteasome (Rechsteiner & Rogers, 1996; Yaglom *et al*., 1995). The phosphorylation of Cln3 is dependent on Cdc28 kinase activity, which negatively regulates Cln3 stability (Yaglom *et al*., 1995).

Upon deletion of *CLN3*, cells have an elongated G_1 phase duration and a delay in *CLN1* and *CLN2* expression, indicating Cln3 functions upstream of Cln1 and Cln2 (Dirick & Nasmyth, 1991; Stuart & Wittenberg, 1995). Overexpression of *CLN3* results in a short G_1 phase and increases expression of transcription factor *SWI4* (Dirick *et al*., 1995; Tyers *et al*., 1993)

Transcription factors Swi4 and Swi6

The transcription factor Swi4 can bind to Swi6 and form the transcriptional activator complex SBF (Swi4/Swi6-dependent cell cycle box Binding Factor) (Andrews & Moore, 1992; Sidorova & Breeden, 1993). The SBF complex is able to bind to SCB elements (Swi4/Swi6-dependent Cell cycle Box) in the promoter of several G_1 specific genes like *CLN1*, *CLN2* and many genes involved cell wall biogenesis (Breeden & Mikesell, 1994; Cross *et al*., 1994; Harrington & Andrews, 1996). DNA-binding protein Swi4 can only bind to SCB elements when Swi6 interacts to its C-terminal domain, thus relieving autoinhibition of the Swi4 DNA-binding domain (Baetz & Andrews, 1999).

The Swi4 protein remains in the nucleus throughout the cell cycle (Baetz & Andrews, 1999), whereas Swi6 is localised in the nucleus only during the G_1 phase, being

cytoplasmic during the rest of the cell cycle (Sidorova *et al*., 1995; Taba *et al*., 1991). The localisation of Swi6 is regulated by phosphorylation at serine 160 from late G_1 phase until late M phase (Sidorova *et al*., 1995). *In vivo* footprinting studies and chromatin immunoprecipitation experiments have shown that the SBF complex is bound to SCB elements in the late M and the G_1 phase (Harrington & Andrews, 1996; Koch *et al*., 1996). This indicates that a secondary event is required before transcriptional activation occurs in late G_1. This activation step of the SBF complex is dependent on Cln3 / Cdc28 kinase activity at Start, but no direct activation of SBF by Cln3 / Cdc28 kinase activity has been detected (Dirick *et al*., 1995; Stuart & Wittenberg, 1995).

The MAP kinase Slt2 (see paragraph MAP kinase pathways) is activated at the end of the G_1 phase and is able to phosphorylate both Swi4 and Swi6 of the SBF complex in vitro (Gray *et al*., 1997; Madden *et al*., 1997; Marini *et al*., 1996; Mazzoni *et al*., 1993; Zarzov *et al*., 1996). Upon activation by phosphorylation, the SBF complex activates transcription of (among others) the cyclins *CLN1*, *CLN2*, *PCL1* and *PCL2* at the end of the G_1 phase (Breeden & Mikesell, 1994; Cross *et al*., 1994; Harrington & Andrews, 1996; Madden *et al*., 1997).

The cyclins Cln1 and Cln2

The cyclins *CLN1* and *CLN2* are expressed once cells pass restriction point Start in the G_1 phase (Price *et al*., 1991; Tyers *et al*., 1993; Wittenberg *et al*., 1990). Upon binding of Cln1 and Cln2 to Cdc28, several G_1- related processes are inhibited and entry into the S phase is initiated (Levine *et al*., 1996). For entry of the S phase, the Sic1 protein is degraded to release Clb5, 6 / Cdc28 activity (Schwob *et al*., 1994; Verma *et al*., 1997). The destruction of Sic1 by ubiquitination and degradation by the proteasome is an essential function of the late-G_1 cyclins (Schneider *et al*., 1996; Verma *et al*., 1997). Cells deleted for the three G_1 cyclins arrest in G_1, but additional deletion of Sic1 renders cells viable (Schneider *et al*., 1996; Tyers, 1996). Furthermore, Sic1 overexpression is lethal in a *cln1*, *cln2* deletion strain, showing the requirement of degradation of Sic1 at the end of the G_1 phase (Tyers, 1996). Cln1 and Cln2 seem also directly involved in the initiation of bud formation, as cells deleted for both cyclins require Bud2, a protein involved in bud site selection, for viability (Benton *et al*., 1993; Cvrckova & Nasmyth, 1993).

The inhibitory action of Cln1- and Cln2-regulated Cdc28 kinase activity includes inhibition of pheromone signal transduction by repressing the Ste20 protein of the mating-pheromone response pathway, which makes cells susceptible for mating only during the G_1 phase (Oehlen & Cross, 1998). Furthermore, the late-G_1 cyclins are

involved in filamentous growth and are required for pseudohyphal growth and haploid invasive growth (Loeb *et al*., 1999).

Other regulatory mechanisms in the G_1 phase

The regulation of cell cycle processes in *S. cerevisiae* is a complex mechanism. In addition to the main molecular mechanism described above, numerous other molecules and signaling pathways are involved in coordinating the timing of cell cycle progression and activating other cell cycle regulated pathways.

Next to the central CDK Cdc28, other CDKs also have functions through the cell cycle and have their own activating cyclins. The CDK Pho85 has 51% homology to Cdc28 and is activated at the end of the G_1 phase by the cyclins Pcl1 and Pcl2 (Espinoza *et al*., 1994; Poon & Hunter, 1995). Upon deletion of *CLN1* and *CLN2*, Pho85 kinase activity is essential for progression through the G_1 phase (Poon & Hunter, 1995). Furthermore, Pho85 is able to phosphorylate Sic1 in vitro and may therefore have a function in Sic1 degradation (Nishizawa *et al*., 1998). As described for the different cyclins, the CDKs of *S. cerevisiae* also have different functions as well as overlapping functions.

A variation on the SBF complex also regulates transcription of important genes during the cell cycle. The Swi6 protein can bind to Mbp1 to form the MBF complex (Mbp1/Swi6-dependent cell cycle box Binding Factor) (Dirick *et al*., 1992; Koch *et al*., 1993). This complex can bind to MCB elements (Mlu1-dependent Cell cycle Box) in the promoter region of several genes involved in DNA synthesis, like *CLB5*, *CLB6* and *POL1* (Epstein & Cross, 1992). To make it even more complicated, the SBF complex is able to bind to genes containing MCB elements, while the MBF complex can activate transcription of SCB regulated genes (Taylor *et al*., 2000). Also here, the different protein complexes have overlapping functions next to specific activities.

Furthermore, there are several signal transduction pathways that influence the cell cycle progression. Apart from several MAP kinase pathways described in the next paragraph, other pathways also influence progression through the cell cycle, like the cAMP/PKA pathway (Hartwell, 1994; Thevelein & de Winde, 1999; Tokiwa *et al*., 1994). Addition of glucose to cells increases both the cAMP level and the level of Cln3 (Hall *et al*., 1998). However, glucose-induced cAMP/PKA activity does not specifically induce Cln3 protein synthesis, but increases the total protein synthesis rate (Hall *et al*., 1998). Rather, a basal activity of the cAMP/PKA pathway seems to be required for cell cycle progression under normal growth conditions (Anghileri *et al*., 1999; Baroni *et al*., 1994; Hubler *et al*., 1993; Markwardt *et al*., 1995). Furthermore, overexpression of Cln3 can bypass the requirement for cAMP to pass Start, indicating that the cAMP/PKA pathway is a non-essential intermediate in regulation of the Cln3 protein level in response to glucose

(Hall *et al*., 1998). However, other reports describe a function of the cAMP/PKA pathway in late G_1 in cell cycle progression, independent of protein synthesis (Hubler *et al*., 1993; Timblin *et al*., 1996). The mode of action of the cAMP/PKA pathway in G_1 phase progression remains to be identified.

Cell signaling: the MAP kinase pathways

Cells can quickly adapt to changes in the environment by rapidly responding signalling pathways. Among these signal transduction pathways, the mitogen-activated protein kinase (MAPK) cascades play an important role (Herskowitz, 1995; Levin & Errede, 1995).

The MAPK pathways generally contain three protein kinases: a MAP kinase, a MAP kinase kinase (MAPKK or MEK) and a MAP kinase kinase kinase (MAPKKK or MEKK) (Cobb & Goldsmith, 1995; Cooper, 1994; Marshall, 1994). Upon activation of the cascade, the MEKK activates MEK by phosphorylation of both a serine and threonine residue. The activated MEK in turn phosphorylates MAPK on a threonine and tyrosine residue, thereby activating the kinase activity. Five different MAPK pathways have been

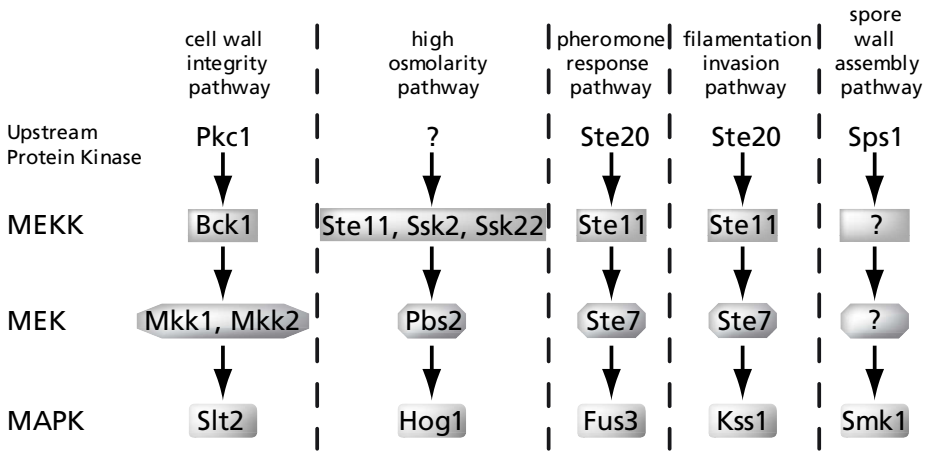


Figure 5: **The MAP kinase cascades of *S.cerevisiae.*** The MAP kinase pathways consist of a MEKK (MAP kinase kinase kinase), a MEK (MAP kinase kinase) and a MAP kinase, which are subsequently phosphorylated upon activation. Five different MAPK pathways have been identified in *S.cerevisiae*, although not all components are known (question marks). All pathways have a unique MAP kinase, but other components may be shared between the different pathways.

identified in *S. cerevisiae*: the pheromone response pathway, the filamentation/invasion pathway, the high osmolarity pathway, the cell wall integrity pathway and the spore wall assembly pathway (figure 5; Gustin *et al*., 1998). Of these MAPK pathways, the spore wall assembly pathway is not present in growing cells.

Two MAPK pathways are involved in cell division cycle arrest in the G_1 phase and initiation of cell development. The pheromone pathway is activated in haploid cells on exposure to the opposite mating factor (Bardwell *et al*., 1994). Activation of the MAP kinase Fus3 of this pathway results in phosphorylation of inhibitor Far1, which results in down-regulation of Cln/ Cdc28 activity and initiation of mating (Jeoung *et al*., 1998; Tyers & Futcher, 1993). Upon nitrogen starvation, the filamentation / invasion pathway is activated. Cells arrest in the G_1 phase and initiate pseudohyphal growth (diploid cells) or invasive growth (haploid cells) (Gimeno *et al*., 1992).

The other two MAPK pathways are involved in the regulation of progression through the normal cell division cycle. The high osmolarity growth (HOG) pathway is activated when cells adapt to hyperosmotic stress and results in a G_1 cell cycle delay (Ferrigno *et al.*, 1998; Brewster *et al.*, 1993; Belli *et al.*, 2001). The activity of the Cln3 / Cdc28 complex is downregulated by HOG activity and the *CLN1* and *CLN2* transcript levels are decreased (Belli *et al*., 2001). Furthermore, the HOG pathway functions upstream of transcription factors Msn2 and Msn4 (Rep *et al*., 2000), which are required for accumulation of the reserve carbohydrates trehalose and glycogen during stress conditions (Parrou *et al*., 1997; Zahringer *et al*., 2000).

The only MAP kinase pathway positively regulating cell cycle progression is the cell wall integrity pathway, or PKC pathway (Irie *et al*., 1993; Lee *et al*., 1993). Activation of the PKC pathway by nutrient addition results in phosphorylation of transcription complex SBF and progression through the G_1 phase as described before (Gray *et al*., 1997; Madden *et al*., 1997; Marini *et al*., 1996; Mazzoni *et al*., 1993; Zarzov *et al*., 1996). The PKC pathway thus mediates cell cycle-regulated transcription of genes involved in cell wall synthesis and integrates cell-wall growth with the cell cycle at Start. Other functions of the PKC pathway include responses to heat stress, hypotonic stress and mating pheromone (Gustin *et al*., 1998).

Reserve carbohydrates and the cell cycle

During growth under external stress or limited nutrient supply, cells elongate their cell cycle and start accumulating reserve carbohydrates (Hottiger *et al*., 1987; Lillie

& Pringle, 1980; Parrou *et al*., 1997). Elongation of the cell cycle mainly influences the G_1 phase duration, whereas the S, G_2 and M phase remain relatively constant (Carter & Jagadish, 1978; Johnston & Singer, 1980). Upon elongation of the cell cycle, the reserve carbohydrates trehalose and glycogen are accumulated during the G_1 phase and degraded at Start (Sillje *et al*., 1997). Although the mechanism that regulates reserve carbohydrate metabolism during the cell cycle is yet to be identified, recent findings indicate that the transcription factors Msn2 and Msn4 may be involved.

Msn2 and Msn4 are required for normal accumulation of trehalose and glycogen during stress (Parrou *et al*., 1997; Zahringer *et al*., 2000). Upon stress, the transcription factors Msn2 and Msn4 become localized in the nucleus by reduced activity of the TOR signaling pathway (Beck & Hall, 1999). Another signaling pathway upstream of Msn2/4 is the cAMP/PKA pathway, as activation of the pathway negatively regulates the nuclear localization of Msn2/4 and the accumulation of reserve carbohydrates (Gorner *et al*., 1998; Hottiger *et al*., 1989; Van Dijck *et al*., 2000). However, hyperactivation of the cAMP/PKA pathway in rapamycin-treated (TOR-inactivated) cells does not inhibit carbohydrate accumulation, indicating that the TOR signaling pathway is the main regulator of Msn2/4 function (Barbet *et al*., 1996). In the nucleus, the transcription factors Msn2 and Msn4 bind to stress response (STRE) elements *in vitro* and *in vivo* (Gorner *et al*., 1998; Martinez-Pastor *et al*., 1996; Schmidt *et al*., 1997). Several functional STRE elements (CCCCT) are found in the promoter region of *TPS1* (coding for trehalose synthetase) and *GSY1* and *GSY2* (coding for glycogen synthetase) (Moskvina *et al*., 1998; Winderickx *et al*., 1996). Thus, the TOR signaling pathway seems to be involved in both cell cycle progression and reserve carbohydrate accumulation. As described previously, activation of the TOR pathway results in translation initiation of early-G_1 cyclin Cln3, whereas deactivation of the TOR signaling pathway induces reserve carbohydrate accumulation and G_1 phase elongation. The TOR signaling pathway may thus link cell cycle progression and reserve carbohydrate accumulation.

Trehalose and glycogen metabolism

During periods of external stress, cells are able to accumulate the reserve carbohydrates trehalose and glycogen. The accumulation of the disaccharide trehalose is catalysed by a protein complex consisting of four different subunits: Tps1, Tps2, Tps3 and Tsl1 (figure 6; Reinders *et al*., 1997). The trehalose-6-phosphate subunit (Tps1) produces trehalose-6-phosphate from glucose-6-phosphate and UDP-glucose. Trehalose-6-phosphate is dephosphorylated by trehalose-6-phosphatase (Tps2) to form

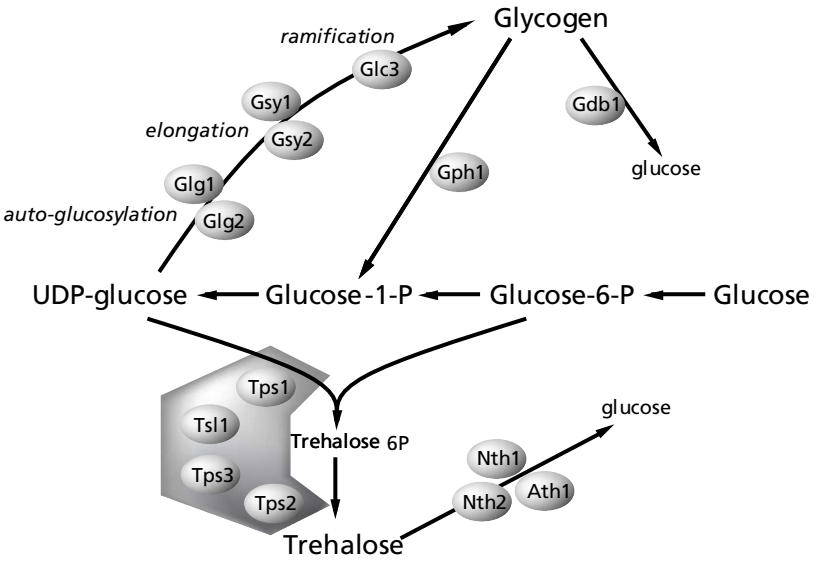


Figure 6: **Metabolic pathways of trehalose and glycogen in *S.cerevisiae.*** Accumulation of the disaccharide trehalose is catalysed by trehalose synthase (Tps1) and trehalose-6-phosphatase (Tps2), which form a complex with the regulatory proteins Tps3 and Tsl1. Degradation of trehalose is catalysed by neutral trehalase (Nth1, Nth2) and acid trehalase (Ath1). Glycogen synthesis is initiated by auto-glucosylation of glycogenin (Glg1, Glg2), elongation by glycogen synthase (Gsy1, Gsy2) and ramification by the transglucosidase Glc3. Degradation of the polysaccharide glycogen is catalysed by glycogen phosphorylase (Gph1) and debranching enzyme Gdb1.

trehalose. The Tps3 and Tsl1 proteins are two regulatory subunits, involved in stabilization of the complex (Bell *et al*., 1998; Reinders *et al*., 1997). The trehalose synthesis complex can be activated by the substrates glucose-6-phosphate and UDP-glucose in vitro. Furthermore, fructose-6-phosphate is a potent activator of this complex, whereas Pi is a strong inhibitor of trehalose-6-phosphate synthase activity (Bell *et al*., 1998; Londesborough & Vuorio, 1993; Vandercammen *et al*., 1989). The activity of the Tps-complex does not seem to depend on activity of the cAMP/PKA pathway, as alteration of cAMP levels in crude extracts or cAMP-altered mutants showed a constant trehalose-6-phosphate synthase activity (Arguelles *et al*., 1993; Vandercammen *et al*., 1989).

Hydrolysis of trehalose is performed by neutral trehalase (encoded by *NTH1* and *NTH2*) and acid trehalase (encoded by *ATH1*) (figure 6; Destruelle *et al*., 1995; Kopp *et al*., 1993; Londesborough & Varimo, 1984; Wolfe & Lohan, 1994). The neutral

trehalase is the major trehalose-mobilizing enzyme, as deletion of *NTH1* leads to complete loss of measurable trehalase activity (Nwaka *et al*., 1995). Neutral trehalase is activated by phosphorylation of serine residues in the N-terminal part of the protein by the cAMP/PKA pathway (Wera *et al*., 1999).

The synthesis of the polymer glycogen involves initiation, elongation and ramification steps (figure 6). Initiation of glycogen synthesis is performed by auto-glucosylation of the glycogenin proteins Glg1 and Glg2 (Cheng *et al*., 1995). The short α (1,4)-glucosyl chain formed on glycogenin is elongated into linear chains of glycogen by glycogen synthase, encoded by *GSY1* and *GSY2* (Farkas *et al*., 1991). The activity of the main glycogen synthase Gsy2 is regulated by phosphorylation (deactivation) at serine and threonine residues on the C-terminus (Hardy & Roach, 1993). Recombinant Gsy2 can be phosphorylated at Ser-654 and Thr-667 by the CDK Pho85, which is activated at the end of the G_1 phase (Huang *et al*., 1998). Deletion of Pho85 results in hyperaccumulation of glycogen and can suppress the glycogen storage defect of *snf1* cells (Huang *et al*., 1996), indicating a role for Pho85 in glycogen mobilization during cell cycle progression. Several studies have indicated that activation of glycogen synthase is related to the levels of glucose-6-phosphate and cAMP (Francois & Hers, 1988; Francois *et al*., 1988; Hardy *et al*., 1994). However, no direct phosphorylation of glycogen synthase by PKA was observed (Hardy & Roach, 1993) and the direct or indirect mode of action of glucose-6-phosphate on glycogen synthase activity remains to be identified (Francois & Parrou, 2001).

The ramification of elongated α (1,4)-chains is catalysed by amylo (1,4)$\rightarrow$(1,6)-transglucosidase, encoded by *GLC3* (Rowen *et al*., 1992). This branching enzyme transfers 6 to 8 residues from the end of a linear chain to create a α (1,6)-linkage to an adjacent chain.

Degradation of glycogen is performed by glycogen phosphorylase (encoded by *GPH1*; releasing glucose-1-phosphate from linear α (1,4)-glucosidic bonds) and by debranching through (1,4)-glucanotransferase and (1,6)-glucosidase activity (by activity of Gdb1). Glycogen phosphorylase activity is increased upon phosphorylation by the cAMP/ PKA pathway, which functions antagonistically with protein kinase Snf1 (Francois & Parrou, 2001). Furthermore, glucose-6-phosphate acts as an inhibitor of glycogen phosphorylase *in vitro* (Lin *et al*., 1996; Wingender-Drissen & Becker, 1983).

Outline of this thesis

The mechanism of cell cycle progression is mostly studied under optimal growth conditions, although most cells only encounter good growth conditions during a short time of their life cycle. Moreover, most of the time in the life cycle of micro-organisms is spend in a resting phase, waiting for environmental conditions to improve. During such periods of prolonged starvation, the cell is able to survive by utilising energy-reserves that were accumulated in the period just before nutrients were depleted. The yeast *Saccharomyces cerevisiae* is able to accumulate the carbohydrates trehalose and glycogen during periods of nutrient limitation, when progression through the cell cycle is reduced. We studied the major checkpoint in the cell cycle of *S. cerevisiae* called 'Start' under limited nutrient supply and the influence of nutrients on cell cycle progression and carbohydrate accumulation under well-controlled growth conditions.

In chapter 2, cell cycle progression was studied in cells grown at different growth rates in fed-batch cultures. It was shown that the MAP kinase Slt2 is phosphorylated at the end of the G_1 phase of cells with a short G_1 phase. Overexpression and deletion studies showed that phosphorylation of Slt2 results in an increase in cell cycle progression by reducing the duration of the G1 phase. In chapter 3, we show that cells with a long G_1 phase duration in fed-batch cultures accumulate the carbohydrates trehalose and glycogen. By using nitrogen-limited continuous cultures, it was shown that the amount of accumulated carbohydrates is dependent on the growth rate of the cell and not to the glucose flux or external glucose concentration. Whereas trehalose is only accumulated at a G_1 phase duration of 5 hours and more, glycogen accumulation gradually increases at decreasing growth rates. In chapter 4, the function of trehalose and glycogen in cell cycle progression and cell viability is described. It was shown that trehalose and glycogen accumulation is not required for cell cycle progression at low growth rates, although accumulation of carbohydrates under these growth conditions induces cell cycle progression upon addition of glucose. Furthermore, we show that both trehalose and glycogen function as reserve carbohydrates under carbon starvation. In chapter 5, the results described in this thesis are discussed in a broader perspective.

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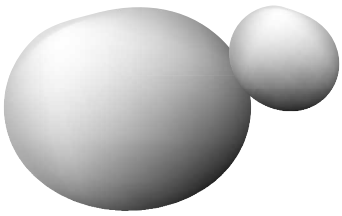
Chapter 2

Phosphorylation of the MAP kinase Slt2 induces cell cycle progression in *Saccharomyces cerevisiae*.



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ABSTRACT

Commitment to progress through the cell division cycle of the yeast *Saccharomyces cerevisiae* is regulated in late G_1 phase, at Start. One of the main determinants for progression through Start is the availability of nutrients. During growth on poor media, the S-, G_2- and M-phase duration remain relatively constant, whereas the G_1-phase duration may vary largely. We used galactose-limited fed-batch cultures, to manipulate the duration of the G_1 phase under constant external conditions. We show that the G_1 phase duration is short at high galactose consumption rates, whereas the G_1 length is strongly increased at low galactose consumption rates. The Slt2 protein was phosphorylated at the end of the G_1 phase of cells with short G_1 phase duration, whereas no phosphorylated Slt2p was detected in cells with an elongated G_1 phase. The relation between Slt2 phosphorylation and the length of the G_1 phase was also observed in an *slt2* deletion strain and a strain overexpressing epitope-tagged *SLT2*. Furthermore, overexpression of *CLN3* resulted in a strong decrease in the G_1 phase duration, concomitant with a high level of Slt2 phosphorylation. These results indicate that progression through the G_1 phase is induced by phosphorylation of Slt2 in late G_1.

INTRODUCTION

The main cell-cycle checkpoint in *Saccharomyces cerevisiae* is located at the end of the G_1 phase at a point called Start (Hartwell *et al*., 1974). Upon passage through Start, the cells are committed to finish the cell cycle. The decision to progress through the cell cycle is strongly affected by environmental conditions like nutrient supply. On nutrient depletion, the G_1-phase lengthens until cells eventually arrest in the G_1-phase (Pringle, 1981). During growth on poor media, the S-, G_2- and M-phase duration remain relatively constant, whereas the G_1-phase duration may vary largely (Carter & Jagadish, 1978; Johnston & Singer, 1980).

Progression through the G_1 phase of the cell cycle is mainly regulated by the activity of the cyclin-dependent kinase Cdc28 (reviewed in Mendenhall & Hodge, 1998; Nasmyth, 1993; Reed, 1992). This protein kinase can be activated by association to one of the G_1 cyclins; Cln1, Cln2 or Cln3. The first cyclin to activate Cdc28 in the G_1 phase is Cln3 (Cross, 1990; Dirick *et al*., 1995; Stuart & Wittenberg, 1995; Tyers *et al*., 1993). The amount of Cln3 protein is dependent on the nutrient availability and is regulated at the level of transcription and translation, as well as on the protein stability (Gallego *et al*.,

1997; Hall *et al*., 1998; Parviz & Heideman, 1998; Polymenis & Schmidt, 1997). The level of Cln3 protein in the cell is increased after glucose addition, giving rise to higher Cln3/Cdc28 kinase activity and a decrease in G_1-phase duration (Hall *et al*, 1998).

Upon induction of Cln3, transcription of transcription factor Swi4 is induced (Tyers *et al*., 1993). When associated to Swi6, the Swi4/Swi6 complex forms the degenes of transcriptional activation complex SBF (Swi cell cycle box [SCB]-Binding Factor) (Cross *et al*., 1994; Nasmyth, 1993). After binding to the promoter-region of *CLN1* and *CLN2*, the SBF complex is phosphorylated by Slt2 (Mpk1), which activates transcription of these late- G_1 genes (Cross *et al*, 1994; Madden *et al*., 1997). Upon association of either Cln1 or Cln2 to Cdc28, the Cdc28 kinase activity drives the cell to the S phase.

Previous studies have shown that upon elongation of the G_1 phase duration, the genes of transcription factors *SWI4* and *SWI6* are expressed relatively early in the G_1 phase, whereas expression of *CLN1* and *CLN2* occurs at the end of the G_1 phase (Sillje *et al*., 1997). The induction of late G_1 cyclin transcription not only depends on the formation of the SBF complex, but also depends on the activity of the Cln3/Cdc28 complex (MacKay *et al*., 2001). The mechanism by which Cln3/Cdc28 activates this transcription remains to be identified (MacKay *et al*., 2001).

The protein Slt2p is a mitogen-activated protein kinase (MAPK) isoform that is part of the PKC/MAP kinase pathway (Gustin *et al*., 1998; Lee *et al*., 1993). This pathway functions to maintain the integrity of the cytoskeleton and cell wall and is activated at the end of the G_1 phase (Gray *et al*., 1997; Marini *et al*., 1996; Mazzoni *et al*., 1993; Zarzov *et al*., 1996). The PKC/MAP kinase pathway is composed of the MAPK kinase kinase Bck1/Slk1, the MAPK kinases Mkk1 and Mkk2 and the MAPK Slt2/Mpk1, which are sequentially phosphorylated upon activation (Mendenhall and Hodge, 1998). The PKC/MAP kinase pathway can be activated by diacylglycerol, of which the production can be induced by Cdc28 activity (Marini *et al*., 1996). Furthermore, Pkc1 is activated by phosphatidylinositol kinase homologue TOR2 via Rho-type GTPase Rho1, to control actin organization needed for polarized growth (Delley & Hall, 1999; Helliwell *et al*., 1998 [B]; Kamada *et al*., 1996; Nonaka *et al*., 1995).

Here we report the role of Slt2 phosphorylation on progression through the G_1 phase under well-defined growth conditions in fed-batch cultures. The duration of the G_1 phase was regulated by controlled addition of galactose to galactose-limited cultures (Sillje *et al*., 1997). Under these growth conditions, the Slt2 protein was phosphorylated at the end of the G_1 phase of cells grown at high galactose consumption rates with a short G_1 phase duration. No phosphorylated Slt2p was detected in the G_1 phase of cells grown at low consumption rates and an elongated G_1 phase duration. Deletion of *SLT2* resulted in an increase in cell cycle duration, whereas overexpression of HA-tagged *SLT2*

resulted in a reduction of the cell cycle duration. These results indicate that Slt2 phosphorylation functions in late G_1 to induce cell cycle progression in response to the nutrient availability. Furthermore, overexpression of *CLN3* resulted in an increase in Slt2 phosphorylation and a strong reduction in the cell cycle duration. A possible function of the Slt2 MAP kinase pathway as a downstream target of Cln3/Cdc28 kinase activity in cell cycle progression is discussed.

MATERIALS AND METHODS

Yeast strains and plasmids

In all experiments the haploid strain CEN-PK 113-7D (*SUC2*, *MAL2-8c*, *MEL*) was used. The SLT2-HA overexpression strain was obtained by transformation of yeast 2μ plasmid YEp352 [*SLT2:HA]* (Kamada *et al*., 1996) into the haploid strain CEN-PK 113-5D (*SUC2*, *MAL2-8c*, *MEL*, *ura3*). The Cln3-overexpression strain was obtained by insertion of PCR amplified Cln3 into the multiple cloning site of yeast 2μ plasmid pYEX-BX (Clontech) and transformation into strain CEN-PK 113-5D. The pYEX-BX plasmid contains the cupper-inducible *CUP1*-promoter, which has a low basal level of expression. No additional cupper was added in our experiments.

The –18 to +1773 fragment of *CLN3* was amplified by PCR using a primer upstream of the *CLN3* starting codon (5'-TATGGATCCTGATACGCTTTCTGTACGATG-3') with a *Bam*HI-site (underlined) introduced and a primer downstream the *CLN3* starting codon (5'-CTATGTCGACTTTGTCGTTTCAGCGAGTTTTC-3') with a *Sal*I-site (underlined) introduced. The PCR-product was digested with *Bam*HI and *Sal*I and inserted in the *Bam*HI/*Sal*I-site of pYEX-BX. The plasmid construct was sequenced to ensure no mutations were generated during PCR amplification.

Growth conditions

All experiments were performed in yeast nitrogen base without amino-acids (6.7 g l^{-1} YNB, Difco) with galactose as carbon source. Asynchronous and synchronous fed-batch cultures were performed in YNB medium with a constant residual galactose concentration of 0.15 mM at an initial cell density of 0.4-1.0 x 10^7 cells ml^{-1}. Galactose was continuously added at rates ranging from 12 fmol $cell^{-1}$ h^{-1} to 20 fmol $cell^{-1}$ h^{-1}. The cell number and the external galactose concentration were monitored throughout the growth. Galactose consumption rates were determined as described previously (Sillje *et al*., 1997). All experiments were performed at least three times and representative results are shown.

Cell synchronization

Centrifugal elutriation was performed essentially as described previously (Woldringh *et al*., 1993), with some modifications. Strain CEN-PK 113-7D was grown exponentially in YNB medium containing 1% galactose at 30^0C and 2 x10^{10} cells were loaded in a 40 ml chamber of a Beckman J-6MI centrifuge (JE-5.0 rotor) at 30 ^{0}C and 2000 rpm. Cells were cultivated in the elutriator chamber in YNB medium containing 1% galactose. Small cells were washed out at a flow rate of 45 ml min^{-1} and newborn daughter cells were collected on ice. The cell size was monitored with a Coulter Multisizer II and the flow rate of the elutriation was adapted to retain a constant cell size of daughter cells.

Preparation of yeast extracts and immunoblot analysis

Cells were collected in a Falcon centrifuge tube, immediately frozen in liquid nitrogen and stored at –80 ^{0}C. The cells were thawed on ice and collected by centrifugation at 4000g for 3 minutes. The pellet was washed in ice-cold water and diluted in PBS containing protease inhibitors (Complete, Boehringer). Equal amounts of cells were lysed by vigorous shaking with 0.45-mm glass beads (BeadBeater, BioSpec Products, Inc.). Cell extracts were separated on 10% SDS-PAGE and transferred to PVDF membrane (Roche, Germany). The membranes were blocked in 2% Protifar (Nutricia, Zoetermeer, The Netherlands) in TBST-buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl and 0.1% Tween20) for 1h at room temperature. Membranes were probed with either anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (New England Biolabs) to detect dually phosphorylated Slt2 (Martin *et al*., 2000). The primary antibody was detected using peroxide-conjugated goat anti-rabbit for detection of phosphorylated Slt2 or rabbit anti-mouse for HA-tag detection (Jackson ImmunoResearch, Pennsylvania, PA). Proteins were visualized by Enhanced Chemiluminescence (Renaissance, Du Punt NEN, Boston, MA).

RESULTS

G_1-phase duration in fed-batch cultures.

Under nutrient limitation, the duration of the cell cycle is elongated by an increase in the duration of the G_1 phase (Carter & Jagadish, 1978; Johnston & Singer, 1980). To study progression through the cell cycle as a function of the nutrient availability, cells of *S. cerevisiae* were grown under well-defined growth conditions in galactose-limited fed-batch cultures (Sillje *et al*., 1997).

Cells were synchronized by centrifugal elutriation and grown in fed batch cultures at different galactose consumption rates. Progression through the cell cycle was monitored by determination of the percentage of budded cells. The end of the G_1-phase was determined as the point at which 50 percent of the cells were budded. The relation between G_1-phase duration and the galactose consumption rate is shown in figure 1.

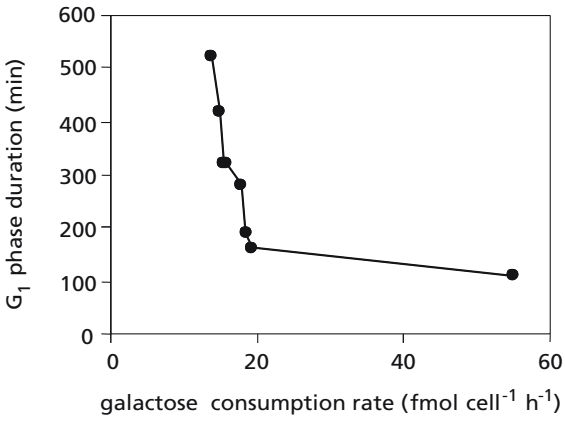

Figure 1: **Correlation between galactose consumption rate and G_1 phase duration.** Cells were synchronized by elutriation and grown at different galactose consumption rates in fed-batch cultures. The G_1 phase duration was determined as the time from inoculation until 50% of the cells were budded.

The G_1-phase duration slowly increased from 110 to 160 minutes when the galactose consumption rate was decreased from 55 fmol galactose $cell^{-1}$ h^{-1} (1% galactose) to 18 fmol galactose $cell^{-1}$ h^{-1}. At lower galactose consumption rates, the length of G_1-phase strongly increased up to 520 minutes at 14 fmol galactose $cell^{-1}$ h^{-1}. Thus, the G_1 phase elongates at decreasing galactose consumption rates, which results in a strong increase in the G_1 phase duration at galactose consumption rates of 18 fmol $cell^{-1}$ h^{-1} and lower. Previously, a similar relation between G_1-phase duration and galactose limitation was described for diploid strain SU32 (Sillje *et al*., 1997).

Phosphorylation of Slt2p during the cell cycle in synchronous fed-batch cultures.

Previous studies have shown that expression of *SWI4* and *SWI6* in the G_1 phase is not the main determinant for timing the transition to S phase (MacKay *et al*., 2001; Sillje *et al*., 1997). The Swi4/Swi6 (SBF) transcription factor is activated upon phosphorylation and can be phosphorylated by the MAP kinase Slt2 in vitro. The possible function of Slt2 in cell cycle progression was studied by determining the amount of phosphorylated Slt2 in cells with a different G_1 phase duration. We monitored the amount of active Slt2 during the cell cycle by using a specific antibody against dually phosphorylated p44/42 MAPK, which specifically recognizes phosphorylated, active Slt2 (Harrison *et al*., 2001; Martin *et al*., 2000). Wild-type cells were synchronized in early G_1 by centrifugal elutriation, grown at different galactose consumption rates and the amount of phosphorylated Slt2p was determined in time (figure 2).

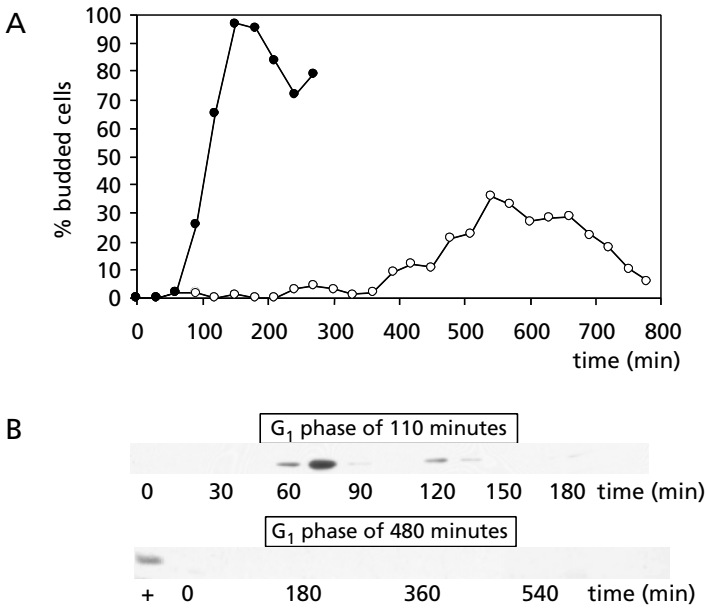

Figure 2: **Phosphorylation of Slt2p during the G_1 phase of the cell cycle as a function of the G_1 phase duration.** Cells were synchronized by elutriation and grown at different galactose consumption rates in fed-batch cultures. A. Budding percentages of cells grown at 55 fmol $cell^{-1}$ h^{-1} (1% galactose) (●) and 14 fmol $cell^{-1}$ h^{-1} (○). B. Phosphorylation of Slt2p during the cell cycle. Samples were taken, cell extracts were separated on SDS-polyacrylamide gel and immunoblotted with anti-phospho-p44/42 MAPK antibodies. As a positive control, a sample from exponentially growing cells was used (+).

Cells grown at a galactose consumption rate of 55 fmol galactose $cell^{-1}$ h^{-1} (1% galactose), had a G_1 phase duration of 110 minutes. Slt2p was phosphorylated in the G_1 phase from 60 to 75 minutes after inoculation, and at a later stage in the cell cycle from 120 to 135 minutes. Probably, the second phosphorylation peak of Slt2 is situated at the morphogenesis checkpoint in the G_2 phase, as was recently described (Harrison *et al*., 2001). At a galactose consumption rate of 15 fmol galactose $cell^{-1}$ h^1, cells had a G_1 phase duration of 480 minutes and no phosphorylation of Slt2 was detected in the G_1 phase. These results show that in cells with a short G_1 phase duration Slt2 is activated at the end of the G_1 phase, whereas no phosphorylated Slt2 was detected in cells with a long G_1 phase duration.

Cell cycle progression of an *slt2* deletion strain.

The amount of phosphorylated Slt2 seems to be related to the duration of the G_1 phase duration, which suggests a role for active Slt2 in cell cycle progression. The possible function of Slt2 in inducing cell cycle progression was studied by growing cells deleted for *SLT2* under growth conditions that induce Slt2 phosphorylation in wild-type cells. Asynchronous cultures of the wild type and *slt2* cells were grown in fed-batch cultures at an initial galactose consumption rate of 25 fmol $cell^{-1}$ h^{-1}. The rate of cell cycle progression was monitored by determination of the amount of newly formed cells after growth (figure 3).

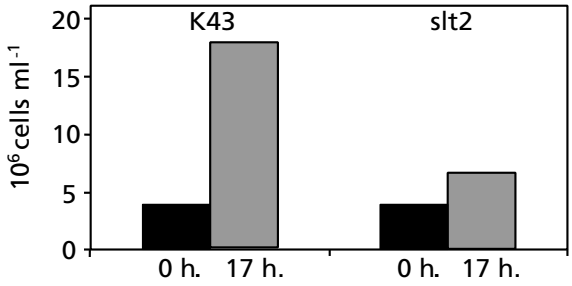

Figure 3: **Effect of *SLT2* deletion on cell cycle progression of cells grown in fed-batch culture.** Exponentially growing cultures of *slt2* cells and wild-type cells (K43) were washed in YNB medium and grown at different galactose consumption rates. Samples were taken from the initial culture (■) and after 17 hours of fed-batch growth (■) at an initial growth rate of 25 fmol galactose $cell^{-1}$-h^1 and cell number was measured with a Coulter Multisizer II.

After 17 hours of fed-batch growth, the amount of wild-type cells in the culture had increased from 3.9 x 10^6 cells ml^{-1} to 17.8 x 10^6 cells ml^{-1}. Under the same

growth conditions, the *slt2* strain produced 6.6 x 10^6 cells ml^{-1} from the same amount of cells. Thus, wild-type cells were able to progress through the cell cycle at a higher rate than cells deleted for *SLT2*. As *slt2* cells were still able to divide, this indicates the Slt2 protein is not essential for cell cycle progression under these growth conditions. Our results indicate that loss of Slt2 function results in an increase in the duration of the cell cycle at high consumption rates.

Overexpression of epitope-tagged *SLT2* in fed-batch cultures.

The increase in phosphorylation of Slt2p prior to budding seems to be related to faster progression through the G_1 phase of the cell cycle. To study the possible positive effect of Slt2 activity on cell cycle progression, HA-epitope tagged Slt2 (Slt2HA) was overexpressed in asynchronous cultures grown at different galactose consumption rates and Slt2 phosphorylation and cell cycle progression were subsequently monitored. Phosphorylation of Slt2p was measured by using a specific antibody that recognizes only the dually phosphorylated, active form of Slt2p (Martin *et al*., 2000; Verma *et al*., 1997).

Overexpression of HA-epitope tagged Slt2p in asynchronous cells at a galactose consumption rate of 55 fmol galactose $cell^{-1}$ h^{-1} (1% galactose) resulted in an increase in Slt2 phosphorylation when compared to the wild type (figure 4A). However, when cells were grown at galactose consumption rate of 15 fmol galactose $cell^{-1}$ h^{-1}, no significant change in the amount of phosphorylated Slt2 was detected upon overexpression of Slt2HA (figure 4A). Therefore, the amount of phosphorylated Slt2 is dependent on the availability of nutrients, as all other growth conditions remained constant. When grown for 25 hours at this low galactose consumption rate, wild-type cells produced 2.4 x 10^6 daughter cells ml^{-1} from an original population of 4.9 x 10^6 cells ml^{-1} (figure 4B). Overexpression of Slt2HA resulted in the production of 3.0 x 10^6 daughter cells ml^{-1} from 4.9 x 10^6 cells ml^{-1} initially. Thus, overexpression of Slt2HA does not result in a significant increase in the amount of daughter cells produced under these growth conditions, indicating that only phosphorylated Slt2 positively influences cell cycle progression.

Overexpression of *CLN3* in fed-batch cultures.

The duration of the G_1 phase is dependent on the nutrient availability and the amount of Cln3p in the cell (Belli *et al*., 2001; Hall *et al*., 1998; Parviz and Heideman, 1998). Previous studies have shown that overexpression of *CLN3* results in increased cell cycle progression by reduction of the G_1 phase duration (Futcher, 1996; Tyers *et al*., 1993). We overexpressed *CLN3* in cells growing in fed-batch cultures to study the effect of *CLN3* on cell cycle progression and the amount of phosphorylated Slt2. Cells were

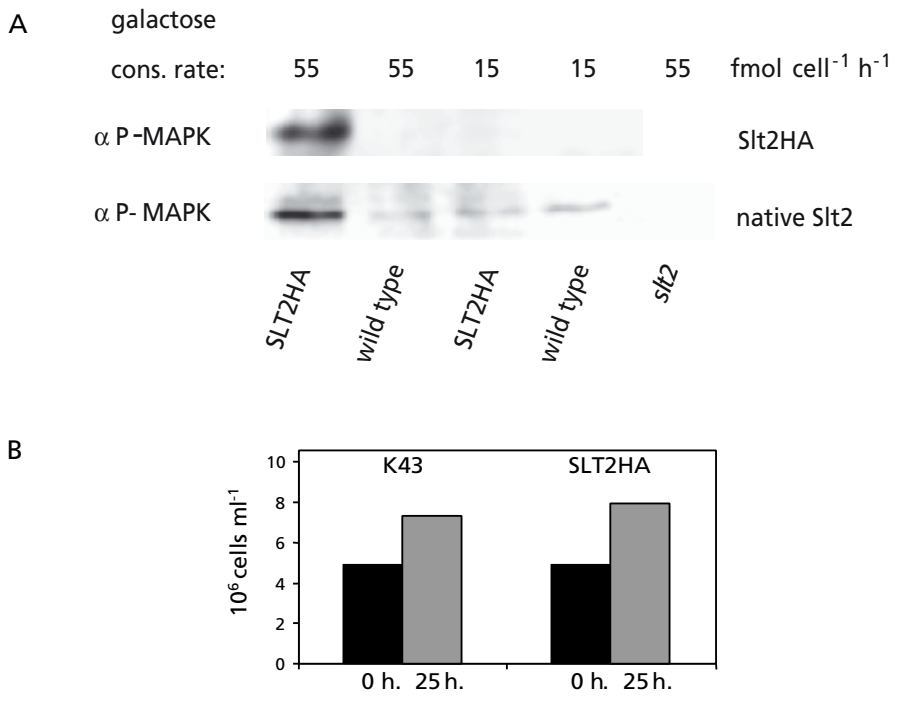

Figure 4: **Effect of overexpression of HA-epitope tagged Slt2 on cell-cycle progression of cells growing at different galactose consumption rates.** Exponentially growing cultures of cells overexpressing HA-epitope tagged *SLT2* (SLT2HA) and wild-type cells (K43) were washed in YNB medium and grown at different galactose consumption rates. *A.* After 6 hours of growth at 15 and 55 fmol galactose cell^{-1} h^{-1}, samples from both strains were taken and separated on SDS-polyacrylamide gel. Immonoblot analysis was performed with anti-phospho-p44/42 MAPK antibodies [αPhospho MAPK]. As a negative control, a sample from the *slt2*-deletion mutant was used. *B.* Samples were taken from the initial cultures (■) and after 25 hours of fed-batch growth (■) at an initial growth rate of 15 fmol galactose cell^{-1}h^{-1} and the cell number was measured with a Coulter Multisizer II.

grown in fed-batch cultures at different galactose consumption rates and cell cycle progression was monitored by comparing the cell number after growth with the initial culture and the amount of phosphorylated Slt2 was determined.

At high galactose consumption rates, a weak band of phosphorylated Slt2 was detected in samples from wild-type cells, while *CLN3* overexpression resulted in a strong increase in Slt2 phosphorylation (figure 5A). At low galactose consumption rates a

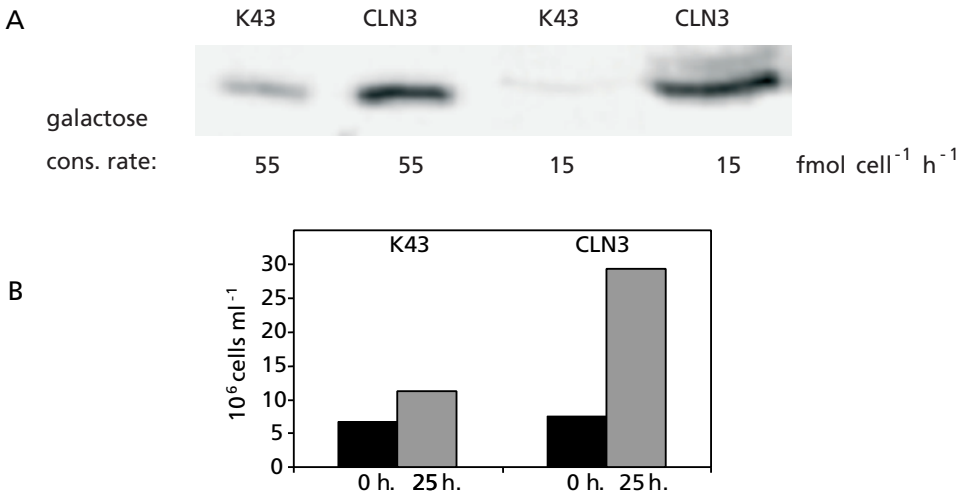

Figure 5: **Effect of *CLN3* overexpression on Slt2 phosphorylation and cell-cycle progression of cells growing at different galactose consumption rates.** Exponentially growing cultures of *CLN3*-overexpressing cells (CLN3) and wild-type cells (K43) were washed in YNB medium and grown at different galactose consumption rates. *A*. Samples were taken after 6 hours of growth and cell extracts were separated on SDS-polyacrylamide gel. Immonoblot analysis was performed with anti-phospho-p44/42 MAPK. *B*. Samples were taken from the initial culture (■) and after 25 hours of fed-batch growth (■) at an initial growth rate of 15 fmol galactose $cell^{-1}hour^{-1}$ and the cell number was measured with a Coulter Multisizer II.

hardly detectable band of phosphorylated Slt2 was visible in wild-type cells. However, also under these conditions overexpression of *CLN3* caused a strong phosphorylation of Slt2. Thus, overexpression of *CLN3* resulted in a high level of Slt2 phosphorylation at different growth rates.

After growth at a galactose consumption rate of 15 fmol galactose $cell^{-1}$ h^{-1} for 25 hours, wild-type cells had formed 11.3 x 10^6 cells ml^{-1} from an initial population of 6.7 x 10^6 cells ml^{-1} (figure 5B). Overexpression of *CLN3* resulted in the formation of 28.2 x 10^6 cells ml^{-1} from an initial culture of 7.6 x 10^6 cells ml^{-1}. By overexpression of *CLN3*, the amount of daughter cells produced at low galactose consumption rates was drastically increased when compared to wild-type cells. Summarizing, these results show that by increasing the level of *CLN3*, the amount of phosphorylated Slt2 is induced and cell cycle progression is drastically increased.

DISCUSSION

By using fed-batch cultures, the G_1 phase duration of the cell cycle was modulated under well-controlled conditions and the different events throughout the cell cycle were studied in response to limited nutrient availability (Sillje *et al*., 1997). The G_1 phase duration was increased on limited amounts of galactose added to cells in galactose-limited fed-batch cultures.

Previous studies from our lab have shown that in galactose-limited fed-batch cultures the expression of *SWI4* and *SWI6* occurs in the beginning of the G_1 phase of cells with a different G_1 phase duration, whereas *CLN1* and *CLN2* are expressed at the end of the G_1 phase, independent of the G_1 length (Sillje *et al*., 1997). The activation of expression of *CLN1* and *CLN2* is regulated by the Swi4/Swi6 complex SBF, which is present throughout the G_1. The SBF complex can be activated upon phosphorylation by Slt2p in vitro, but the mechanism that triggers rapid induction of transcription of late G_1 cyclins remains to be identified (MacKay *et al*., 2001; Madden *et al*., 1997). Our study shows that a high amount of Slt2 is phosphorylated in late G_1 at high galactose consumption rates, whereas no phosphorylation of Slt2p was observed in the G_1 phase at low galactose consumption rates. These results indicated that an increase in Slt2 activity is involved in inducing rapid progression through the G_1 phase at high consumption rates. Indeed, we found that deletion of *SLT2* resulted in a strong increase in cell cycle duration when compared to the wild type. In agreement with this, cultures of *slt2* have a higher G_1 population than wild-type cells and an increase in cell size, which indicates that *SLT2* deletion results in an increase in the G_1 phase duration (Mazzoni *et al*., 1993). Taken together, our results indicate that Slt2 phosphorylation positively regulates progression through the G_1 phase in response to the availability of nutrients.

Interestingly, overexpression of *SLT2HA* resulted in a strong increase in the amount of phosphorylated Slt2 only at high consumption rates. This indicates that the amount of Slt2 protein may be rate limiting for the total amount of phosphorylated Slt2 when nutrients are abundant. At low galactose consumption rates, the amount of phosphorylated Slt2 was not induced upon overexpression of Slt2HA. Thus, the amount of Slt2 protein is not rate limiting for the level of Slt2 phosphorylation under nutrient-limited growth. This indicates that the amount of Slt2 protein is not rate determining for cell cycle progression under low nutrient supply. Rather, the amount of available nutrients determines the amount of phosphorylated Slt2 under these conditions and thus the duration of the G_1 phase.

One of the main determinants of progression through the G_1 phase is the level

of cyclin Cln3, which is dependent on the amount of nutrients in the medium (Hall *et al*., 1998; Polymenis & Schmidt, 1997). The effect of *CLN3* on cell cycle progression was studied independent of the nutrient availability, by overexpression of *CLN3* in galactose-limited fed-batch cultures. At both high and low consumption rates, overexpression of *CLN3* resulted in an increase in Slt2 phosphorylation. This indicates that Slt2 may be one of the downstream targets of Cln3 in cell cycle progression.

The pathway by which the level of Cln3 may regulate the amount of Slt2 phosphorylation has yet to be determined. Probably, Cln3 induces Slt2 phosphorylation through the PKC signaling pathway (Gustin *et al*., 1998). The activity of the Cln3/Cdc28 complex induces the production of diacylglycerol (DAG), which can activate the PKC pathway in vitro (Marini *et al*., 1996; Ogita *et al*., 1990). Furthermore, both translation of Cln3 and activation of Pkc1 are activated by the TOR pathway (Barbet *et al*., 1996; Helliwell *et al*., 1998 [A]; Helliwell *et al*., 1998 [B]; Schmidt *et al*., 1997). Together with our results, this indicates that cyclin Cln3 may function downstream of the TOR pathway and upstream of the PKC pathway in this cascade. The TOR pathway may therefore up-regulate Cln3 translation in response to nutrients, resulting in induction of the PKC signaling pathway, phosphorylation of Slt2 and progression through the G_1 phase. Further research may elucidate the regulatory mechanism by which Cln3 positively regulates phosphorylation of Slt2.

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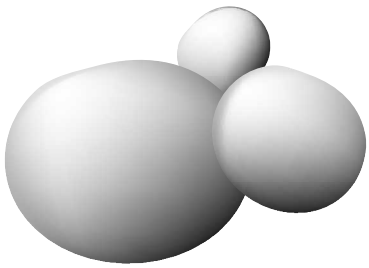
Chapter 3

Trehalose and glycogen levels are determined by the growth rate in *Saccharomyces cerevisiae*.



Submitted for publication

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ABSTRACT

Several factors have been implied as initiator of trehalose and glycogen synthesis, like the glucose flux, the growth rate, the intracellular glucose-6-phosphate levels and glucose concentration in the medium. In this study, the possible relation of these putative initiators to reserve carbohydrate accumulation was studied under well-defined growth conditions in nitrogen-limited continuous cultures. The accumulation of trehalose and glycogen was shown to be dependent on the growth rate applied to the culture, whereas other implicated initiators did not exhibit a correlation with carbohydrate accumulation. Trehalose accumulation was induced at a dilution rate of $D \leq 0.10\ h^{-1}$, whereas glycogen accumulation gradually increased at decreasing growth rates. The growth-rate dependency of trehalose accumulation was supported by studies in cells overexpressing the G_1-cyclin *CLN3*. Trehalose accumulation appeared to be dependent on the duration of the G_1 phase, as trehalose accumulation was induced at a G_1 phase duration of more than 5 hours, in both wild type and *CLN3* overexpressing cells. On the other hand, the amount of accumulated glycogen was reduced by *CLN3* overexpression in a cell-cycle independent manner. A possible regulatory mechanism that links trehalose and glycogen accumulation to the growth rate of cells is discussed.

INTRODUCTION

The reserve carbohydrates trehalose and glycogen are accumulated upon nutrient deprivation and external stress. These non-optimal growth conditions result in a decrease in the growth rate and elongation of the cell cycle duration; mainly caused by an increase in G_1 phase duration (Carter & Jagadish, 1978; Johnston & Singer, 1980; Lillie & Pringle, 1980). Previous studies in fed-batch cultures have shown that trehalose and glycogen are accumulated during the G_1 phase (Sillje *et al*., 1997). Several factors have been implicated as a trigger for initiation of trehalose and glycogen accumulation, like the glucose flux and intracellular glucose-6-phosphate levels (Enjalbert *et al*., 2000; Francois & Parrou, 2001).

Recent studies have identified several possible pathways that may link regulation of synthesis of trehalose and glycogen with progression through the cell cycle at the molecular level. The duration of the G_1 phase in response to nutrient availability is mainly dependent on the activity of the Cln3 / Cdc28 kinase complex (Mendenhall & Hodge, 1998; Nasmyth, 1993; Reed, 1992; chapter 2). The nutrient availability regulates the Cln3 amount in the cell at both the transcriptional and the

translational level. Addition of a glucose or nitrogen source to nutrient-starved cells rapidly increases the *CLN3* level in the cell (Parviz & Heideman, 1998). At the translational level, the phosphatidylinositol kinase homologue TOR2 positively regulates the translation rate of Cln3 upon nutrient addition (Barbet *et al*., 1996). The TOR signalling pathway functions upstream of Msn2 and Msn4; transcription factors that bind to stress response (STRE) elements in promoter sequences (Barbet *et al*., 1996; Beck & Hall, 1999; Martinez-Pastor *et al*., 1996; Schmitt & McEntee, 1996). When the TOR pathway is deactivated, Msn2/4 become localised in the nucleus and transcription of STRE-controlled genes is activated (Gorner *et al*., 1998). The genes encoding trehalose synthase (*TPS1*) and glycogen synthase (*GSY1* and *GSY2*) contain STRE-elements in their promoter region (Moskvina *et al*., 1998; Winderickx *et al*., 1996). Therefore, accumulation of the carbohydrates and one of the main cell cycle regulators seem to be under control of the same pathway. Although these molecular studies implicate a link between the cell cycle and carbohydrate accumulation, no direct evidence has been found that describes this possible link.

In this study, the effect of possible triggers on trehalose and glycogen accumulation was studied under well-defined growth conditions. By using nitrogen-limited continuous cultures, it was shown that the amount of accumulated carbohydrates depends on the growth rate applied to the cell culture. A sharp increase in trehalose accumulation was observed at cell doubling times of more than 8 hours. The accumulation of glycogen increases gradually as the growth rate decreases in wild-type cells. Trehalose accumulation showed a clear dependency on the G_1 phase duration in continuous cultures, in both wild type and *CLN3*-overexpressing cells. The amount of accumulated glycogen was strongly decreased upon *CLN3* overexpression and showed no dependency on the length of the G_1 phase. A possible mechanism for integrating reserve carbohydrate accumulation in cell-cycle progression is discussed.

MATERIALS AND METHODS

Yeast strains and plasmids

The haploid strain CEN-PK 113-7D (*SUC2*, *MAL2-8c*, *MEL*) was used as wild-type strain in all experiments. The *CLN3* overexpression strain was obtained by insertion of PCR amplified *CLN3* into the multiple cloning site of yeast 2μ plasmid pYEX-BX (Clontech) and transformation into strain CEN-PK 113-5D (*SUC2*, *MAL2-8c*, *MEL*, *ura3*). The pYEX-BX plasmid contains the cupper-inducible *CUP1*-promoter, which has a low

basal level of expression. No additional cupper was added in our experiments.

The –18 to +1773 fragment of *CLN3* was amplified by PCR using a primer upstream of the *CLN3* starting codon (5'-TATGGATCCTGATACGCTTTCTGTACGATG-3') with a *Bam*HI-site (underlined) introduced and a primer downstream the *CLN3* starting codon (5'-CTATGTCGACTTTGTCGTTTCAGCGAGTTTTC-3') with a *SalI*-site (underlined) introduced. The PCR-product was digested with *Bam*HI and *Sal*I and inserted in the *Bam*HI/*Sal*I-site of pYEX-BX. The plasmid construct was sequenced to control if mutations were generated by PCR.

Fed-batch growth conditions

All experiments were performed at 30^0C in yeast nitrogen base without amino-acids (6.7 g l^{-1} YNB, Difco) with galactose as carbon source. Synchronous fed-batch cultures were performed in YNB medium with a constant residual galactose concentration of 0.15 mM at a cell density of 1-2 x 10^7 cells ml^{-1}. Galactose was continuously added at rates ranging from 12 fmol $cell^{-1}$ h^{-1} to 20 fmol $cell^{-1}$ h^{-1}. The cell number and the external galactose concentration were monitored throughout the experiment. Galactose consumption rates were determined as described previously (Sillje *et al.*, 1997).

Cell synchronization

Centrifugal elutriation was performed essentially as described previously, with some modifications (Woldringh *et al*., 1993). The yeast strains were grown exponentially in YNB medium containing 1% galactose at 30^0C and 2 x10^{10} cells were loaded in a 40 ml chamber of a Beckman J-6MI centrifuge (JE-5.0 rotor) at 30 ^{0}C and 2000 rpm. Cells were cultivated in the elutriator chamber on YNB medium containing 1% galactose. Small cells were washed out at a flow rate of 45 ml min^{-1}. and newborn daughter cells were collected on ice. The cell size was monitored with a Coulter Multisizer II and the flow rate of the elutriation was adapted to remain a constant cell size.

Continuous culturing.

For continuous culture experiments, cells were grown in a 2 l. BioflоIII chemostat (New Brunswick Scientific; Nijmegen, The Netherlands) connected to a computer controller unit running on Advanced Fermentation Software (New Brunswick Scientific). Cells were inoculated in the medium as described previously (Meijer *et al*., 1998) and a continuous feed was connected after batch growth overnight. The EGLI-medium used in continuous cultures was described previously (Meijer *et al.*, 1998), in which the NH_4^+ concentration was adapted to 1.5 g l^{-1} for nitrogen limitation. In studies

using varying dilution rates under nitrogen limitation, cells were grown at dilution rates (D) ranging from 0.07 up to 0.25 h^{-1} at a constant feed concentration of 200 mM glucose. During studies at constant dilution rates of 0.1 h^{-1} and 0.2 h^{-1}, the glucose feed concentrations ranged from 240 to 420 g l^{-1} (D= 0.1 h^{-1}) and from 140 to 340 g l^{-1} (D= 0.2 h^{-1}). Steady state samples were taken as described previously (Meijer *et al.*, 1998). Additional, samples for metabolite determination were rapidly frozen in cold methanol and isolated as described previously (Gonzalez *et al.*, 1997).

Analysis of sample parameters.

Cell sizes and cell numbers were determined with an electrical particle counter (Coulter Multisizer II). Cell sizes were calculated by calibration with latex beads of known size. For determining budding percentages 200 cells were analysed microscopically. Dry weights were determined by spinning down 10 ml of culture volume in duplicate. Cell pellets were washed twice in water, transferred into pre-weight bottles and dried for at least 12 h at 120^0C before weight determination.

Determination of trehalose and glycogen levels.

Samples of 2 ml were centrifuged for 30 s at maximum speed (Eppendorf centrifuge). The cells were washed in ice-cold water and trehalose and glycogen was extracted from the pellet and the glucose content of both carbohydrates in the samples was determined as described previously (Sillje *et al.*, 1997).

Metabolite concentrations.

External glucose and internal metabolite concentrations were determined essentially as described before (Bergmeyer, 1974). In addition to the sample, enzyme and cofactors, 100 mM imidazole, 10 mM $MgCl_2$ pH7.0 was used as a buffer in all metabolite determinations. The mean values of the duplicate samples had a standard error of the mean (SEM)<10%.

RESULTS

Trehalose and glycogen accumulation in fed-batch cultures.

The carbohydrates trehalose and glycogen are accumulated in batch cultures when nutrients become depleted and cells enter the stationary phase (Lillie and Pringle, 1980). At the onset of carbohydrate accumulation under these growth conditions the nutrient flux rapidly drops, which may therefore be the trigger for initiating

accumulation. The effects of the nutrient flux on trehalose and glycogen accumulation were studied by growing cells at different galactose consumption rates in fed-batch cultures. Cells were synchronised, to maintain a constant cell number and a constant galactose consumption rate per cell throughout the growth. As shown in figure 1, a biphasic correlation was observed between the galactose flux and the amount of carbohydrates accumulated. At galactose consumption rates of 20 fmol galactose $cell^{-1}$ h^{-1} and higher, low amounts of trehalose and glycogen were accumulated, at maximal levels of 6 fmol (glucose) $cell^{-1}$ trehalose and of 18 fmol (glucose) $cell^{-1}$ glycogen. As the galactose consumption rate decreased from 20 to 14 fmol galactose $cell^{-1}$ h^{-1}, trehalose and glycogen accumulation rapidly increased to 38 and 57 fmol

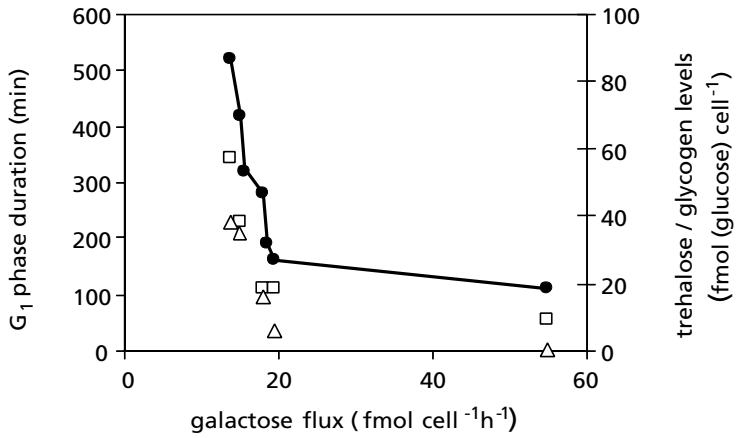

Figure 1: **Correlation between the duration of the G_1 phase, trehalose and glycogen levels and the galactose consumption rate of the cell.**
Cells were synchronised by centrifugal elutriation and grown on minimal medium containing either 1% galactose or in galactose-limited fed-batch cultures at different galactose consumption rates. The maximal levels of trehalose (Δ) and glycogen (□) during the G_1 phase of the cell cycle were determined and plotted against the galactose consumption rate applied. The G_1 phase duration was defined as the time from inoculation until 50% budding was reached. (●) G_1 phase duration (min.).

(glucose) $cell^{-1}$ respectively at 14 fmol galactose $cell^{-1}$ h^{-1}. These results indicate that accumulation of carbohydrates is regulated by the nutrient flux applied to the cells.

As the carbon flux in the cell is decreased, the duration of the G_1 phase of the cell cycle is increased (figure 1). The minimal G_1 phase duration of cells grown in batch culture at 55 fmol galactose $cell^{-1}$ h^{-1} (1% galactose, when galactose in not limited) was

determined at 110 minutes. At decreasing galactose consumption rates, the G_1 phase duration slowly increases to 160 minutes at 20 fmol galactose cell^{-1} h^{-1}. As the galactose flux further decreases, the G_1 phase duration rapidly elongates from 160 to 520 minutes at 14 fmol cell^{-1} h^{-1}. Summarising, cells grown in fed-batch cultures at low carbon fluxes accumulate trehalose and glycogen concomitant with an increase in the G_1 phase duration.

Effects of the carbon flux on trehalose and glycogen accumulation.

In fed-batch cultures, the carbon flux and the growth rate of cells are always correlated, but in nitrogen-limited continuous cultures these parameters can be studied separately (Meijer *et al.*, 1998). The effects of both carbon flux and growth rate on trehalose and glycogen accumulation were therefore studied under these growth

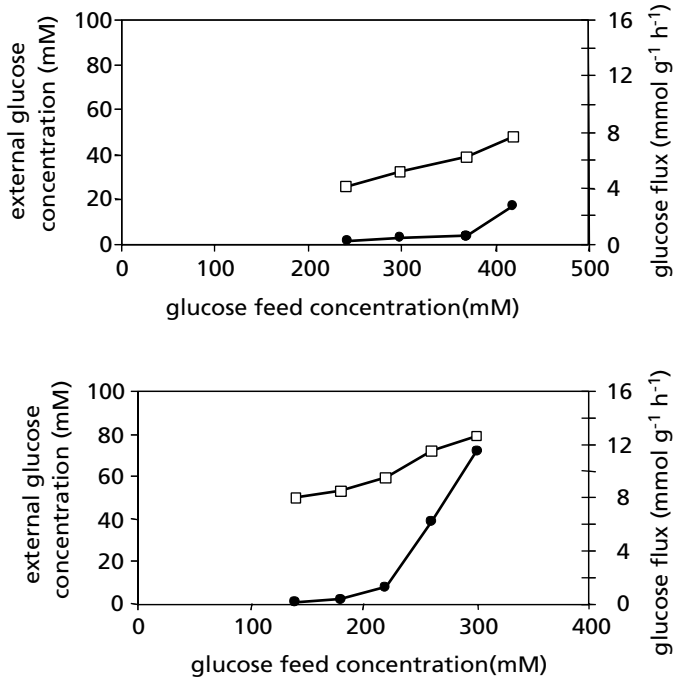

Figure 2: **Glucose flux and external glucose concentration as a function of the glucose feed concentration in nitrogen-limited continuous cultures.**
Cells were cultivated under nitrogen limitation in continuous cultures at a constant dilution rates of D=0.10 h^{-1} (A) and D=0.19 h^{-1} (B). The glucose feed medium increased 240 to 420 g l^{-1} (D= 0.10 h^{-1}) and from 140 to 340 g l^{-1} (D= 0.19 h^{-1}). The glucose flux (□, mmol g^{-1} h^{-1}) and the external glucose concentration (●, mM) were determined at each feed concentration.

conditions. Cells were grown in nitrogen-limited continuous cultures at a constant growth rate and the glucose concentration in the feed medium was varied. At a constant dilution rate of D= 0.1 h^{-1} and feed concentrations from 240 to 420 mM glucose, the external glucose concentration increased from 2 to 17 mM glucose and the glucose flux increased from 4.1 to 7.7 mmol g^{-1} h^{-1} (figure 2A). On applying a dilution rate of D=0.19 h^{-1} and feed concentrations from 140 to 340 mM glucose, the external glucose concentration increased from 1 to 97 mM glucose as the glucose flux increased from 8.0 to 12.6 mmol g^{-1} h^{-1} (figure 2B). Thus, under these growth conditions the glucose flux was varied independent from the growth rate of the culture.

The effect of the growth rate, glucose concentration and the glucose flux on carbohydrate accumulation was studied by determining the amount of trehalose and glycogen in these nitrogen-limited continuous cultures. When grown at a growth rate of D=0.1 h^{-1}, the glucose flux increased from 4.1 to 7.7 mmol g^{-1} h^{-1}, but the trehalose and glycogen accumulation remained at a constant high level at 5 and 110 µg glucose [mg DW]$^{-1}$ respectively (figure 3A). This indicates that the glucose flux is not the trigger for carbohydrate accumulation under these growth conditions. At a higher growth rate of 0.19 h^{-1}, both trehalose and glycogen are accumulated at low levels, although only the trehalose levels remained constant. As the glucose flux increased from 8.0 to 12.6 mmol g^{-1} h^{-1}, trehalose was accumulated at an amount of 0.8 µg glucose [mg DW]$^{-1}$. Also at higher growth rates, the glucose flux did not correlate with the amount of trehalose accumulated, which indicates that the glucose flux is not the trigger for accumulation of trehalose in these continuous cultures. At a growth rate of 0.19 h^{-1} amount of accumulated glycogen decreased from 49 to 14 µg glucose [mg DW]$^{-1}$ as the glucose flux increased from 8.0 to 12.6 mmol g^{-1} h^{-1} (figure 3B). This decrease in glycogen accumulation coincides with a substantial increase in the external glucose concentration from 1 to 97 mM glucose (figure 2A). In contrast to trehalose accumulation, intracellular glycogen levels may be influenced by the glucose flux or the external glucose concentration. Nevertheless our results indicate that the glucose flux is not the main trigger for glycogen accumulation, as constant levels of glycogen are accumulated at the low growth rate independent of the glucose flux.

Previous studies in batch cultures have also suggested that changes in glucose-6-phosphate levels could trigger trehalose and glycogen synthesis. Upon depletion of glucose in the batch cultures, carbohydrates are accumulated concomitant with a drop in glucose-6-phosphate and a drop in growth rate (Enjalbert *et al.*, 2000). In our continuous cultures, the intracellular glucose-6-phosphate concentration remains constant at approximately 7 µmol [mg DW]$^{-1}$ at all growth conditions, whereas the accumulation of trehalose and glycogen decreases at increasing growth rates. It is

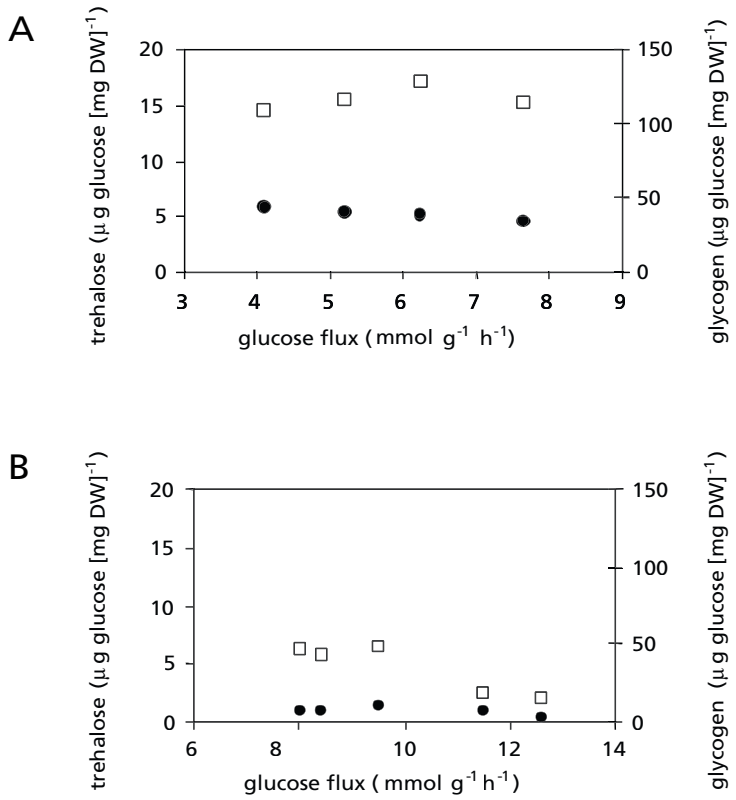

Figure 3: **Trehalose and glycogen accumulation as a function of the glucose flux at constant dilution rates.**
Cells were cultivated under nitrogen limitation in continuous cultures at a constant dilution rates of D=0.10 h^{-1} (A) and D=0.19 h^{-1} (B). The glucose feed medium increased 240 to 420 g l^{-1} (D= 0.10 h^{-1}) and from 140 to 340 g l^{-1} (D= 0.19 h^{-1}). The amount of trehalose (●, µg [mg DW]$^{-1}$) and glycogen (□, µg [mg DW]$^{-1}$) was determined at each dilution rate.

therefore unlikely that glucose-6-phosphate levels regulate the accumulation of trehalose and glycogen.

Trehalose and glycogen accumulation is determined by the growth rate.

The results from both fed-batch cultures and nitrogen-limited continuous cultures show that increases in trehalose accumulation do not correlate with the carbon flux or intracellular glucose-6-phosphate levels. On the other hand, trehalose and glycogen accumulation appear to be accompanied by a decrease in growth rates under all growth conditions. To further investigate this possible relation, we studied trehalose

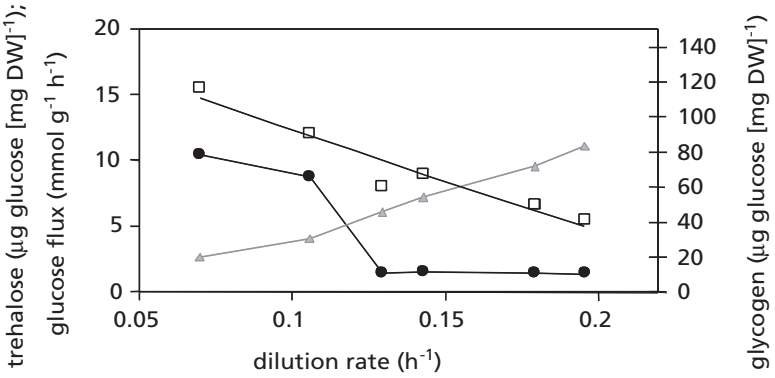

Figure 4: **Trehalose and glycogen accumulation as a function of the dilution rate.**
Cells were cultivated under nitrogen limitation in continuous cultures at increasing dilution rates. The glucose concentration in the feed medium was kept constant at 220 mM glucose. The amount of trehalose (●, µg [mg DW]$^{-1}$) and glycogen (□, µg [mg DW]$^{-1}$) was determined at each dilution rate. (▲) glucose flux (mmol g^{-1} h^{-1}).

and glycogen accumulation as a function of the growth rate in nitrogen-limited continuous cultures (figure 4).

High amounts of trehalose were accumulated in cells grown at dilution rates from 0.07 h^{-1} to 0.095 h^{-1}, at an amount of 10.5 to 8.8 µg glucose [mg DW]$^{-1}$ respectively. At growth rates of D=0.125 h^{-1} and higher, low amounts of trehalose were accumulated (1.4 µg glucose [mg DW]$^{-1}$). Glycogen accumulation gradually decreased from 116 µg glucose [mg DW]$^{-1}$ at D=0.07 h^{-1} to 42 µg glucose [mg DW]$^{-1}$ at D=0.19 h^{-1}. Whereas trehalose was only accumulated at low growth rates, glycogen accumulation gradually decreased as the growth rate increased. These results clearly show that the growth rate applied to the culture determines the amount of accumulated carbohydrates.

Trehalose accumulation is dependent on the G_1-phase duration.

As the growth rate of cells decreases, mainly the G_1 phase duration of the cell elongates and trehalose and glycogen become accumulated (Carter & Jagadish, 1978; Johnston & Singer, 1980; Sillje *et al.*, 1997). The possible relationship between the length of the G_1 phase and the accumulation of carbohydrates was studied by overexpression of *CLN3* in nitrogen-limited continuous cultures. Overexpression of *CLN3* results in a decrease in G_1 phase duration (chapter 2; Tyers *et al.,* 1993) and an increase in the G_2/M phase duration (Futcher, 1996; Tyers *et al*., 1993).

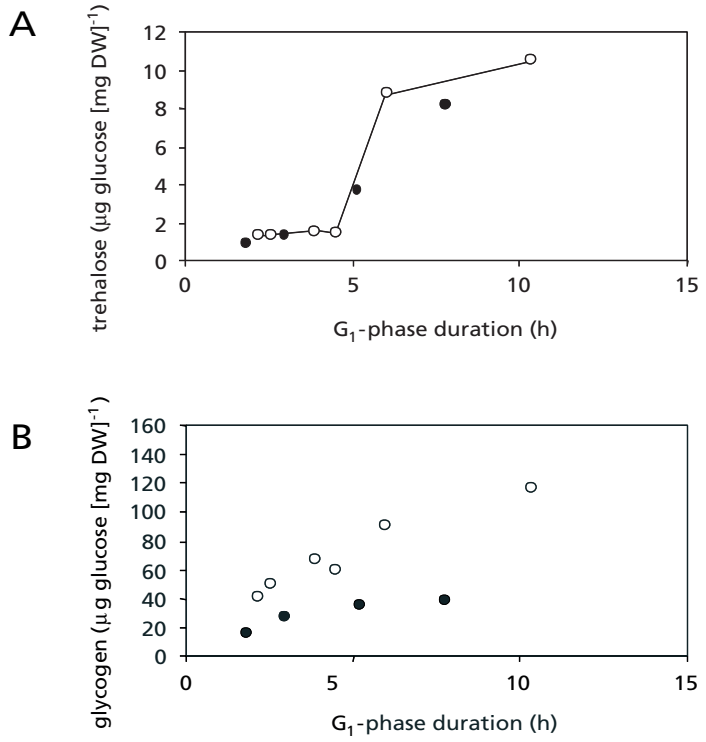

Figure 5: **Trehalose and glycogen accumulation as a function of the G_1 phase duration.**
Wild-type (❍) cells and *CLN3* overexpressing cells (●) were cultivated at increasing dilution rates as described in figure 1 and figure 3. The amount of trehalose (A, µg [mg DW]$^{-1)}$ and glycogen (B, µg [mg DW]$^{-1}$) was plotted against the G_1 phase duration of the cells. The G_1 phase duration was calculated from the percentage of budded cells and the applied dilution rate.

Trehalose and glycogen levels were determined at growth rates from 0.07 h^{-1} to 0.15 h^{-1} and plotted against the G_1-phase duration of *CLN3* overexpressing and wild-type cells. The G_1 phase duration was calculated from the budding percentage of the cells and the applied growth rate (figure 5A, B). The amount of accumulated trehalose was below 2 µg glucose [mg DW]$^{-1}$ at a G_1-phase duration of 4.5 hours and lower. As the G_1-phase duration increases to more than 5 hours, much higher levels of trehalose are accumulated: up to 10.5 µg glucose [mg DW]$^{-1}$ in wild-type cells with a G_1 phase of 10 hours and 8.1 µg glucose [mg DW]$^{-1}$ in *CLN3*-overexpressing cells with a G_1 phase duration of 8 hours. These results show that trehalose accumulation is directly

correlated to the duration of the G_1 phase in both wild type and *CLN3* overexpressing cells.

The glycogen levels show a 2.5 fold decrease upon overexpression of *CLN3* at the same G_1 length as wild-type cells (figure 4B). At a short G_1 phase duration of 2 hours, wild type cells accumulate 42 µg glucose [mg DW]$^{-1}$, whereas *CLN3* overexpressing cells accumulated 16 µg glucose [mg DW]$^{-1}$. As the G_1 phase is elongated to 8 hours, wild-type and *CLN3* overexpressing cells accumulate 105 and 38 µg glucose [mg DW]$^{-1}$ respectively. These results indicate that either glycogen accumulation is not dependent on the G_1 phase duration or that *CLN3* overexpression influences glycogen accumulation directly.

DISCUSSION

Several reports have described that accumulation of trehalose and glycogen coincides with a reduced growth rate of the cell (Kuenzi & Fiechter, 1972; Sillje *et al.*, 1997). However, different factors that coincide with low growth rates in batch culture have been implicated as a trigger for reserve carbohydrate accumulation, like a drop in glucose-6-phosphate levels, a decrease in the glucose flux and depletion of external glucose (Enjalbert *et al.*, 2000; Francois & Parrou, 2001). By using nitrogen-limited continuous cultures, the influence of the individual factors on accumulation of trehalose and glycogen was studied, as the external conditions could be kept constant at different growth rates.

Here, we show that trehalose- and glycogen-accumulation are directly related to the growth rate of wild-type cells. Moreover, the glucose flux, glucose-6-phosphate levels and the external glucose concentration do not correlate to reserve carbohydrate accumulation under different growth conditions. Trehalose accumulation is directly related to the G_1-phase duration of the cells and is induced at a G_1 phase duration of 5 hours and more. In agreement with this, recent observations indicate that heat- or osmo-shock treatment of cells results in a decrease in growth rate, concomitant with accumulation of trehalose (R. Verwaal, personal communication). This suggests that as a general stress response to non-optimal environmental conditions, the growth rate of cells is reduced and accumulation of trehalose is initiated.

The accumulation of glycogen is differently regulated then trehalose accumulation. In contrast to trehalose, glycogen levels are influenced by overexpression of G_1 cyclin *CLN3* in continuous cultures. The amount of Cln3 in the cell regulates the duration of the G_1 phase and is therefore one of the main determinants of the growth

rate of the wild-type cells (Hall *et al*., 1998; Parviz & Heideman, 1998; Polymenis & Schmidt, 1997). In wild-type cells, glycogen levels gradually increase as the growth rate decreases, whereas overexpression of *CLN3* results in a strong decrease in the glycogen level. These results indicate that the accumulation of glycogen is dependent on the amount of Cln3 in the cell. Previous studies showed that Cln3 functions upstream of the SBF transcription factor, which regulates transcription of amongst others cyclins *PCL1* and *PCL2* (Espinoza *et al*., 1994; Measday *et al*., 1997; Tyers *et al.*, 1993). These cyclins can activate the CDK Pho85 at the end of the G_1 phase of the cell cycle and initiate cell cycle progression (Measday *et al.*, 1997; Tyers *et al.*, 1993). Deletion of Pho85 results in hyperaccumulate glycogen, whereas Pho85 activity is also involved in phosphorylation of glycogen phosphorylase and glycogen degradation (Timblin *et al*., 1996; Wilson *et al*., 1999). Possibly, overexpression of *CLN3* results in an increase in glycogen degradation by induction of Pho85 activity.

The mechanism that couples the growth rate to the accumulation of carbohydrates has yet to be identified, but our results imply a link with cell-cycle regulatory pathways. A good candidate for such a regulatory function is the TOR pathway. The TOR pathway controls STRE-dependent expression by regulating the localisation of transcription factors Msn2 and Msn 4 (Gorner *et al.*, 1998). The promoters of the genes encoding trehalose and glycogen synthetase contain several STRE-elements that are negatively regulated by TOR activity (Gorner *et al.*, 1998; Moskvina *et al.*, 1998; Winderickx *et al.*, 1996). Inhibition of TOR activity results in a reduction in translation of Cln3, cell cycle arrest early in the G_1 phase and accumulation of carbohydrates (Barbet *et al.*, 1996). Together with our results, this indicates that the TOR kinase pathway may be involved in linking the growth rate of cells to the amount of carbohydrate accumulation.

Recent observations indicate that not only trehalose and glycogen synthase, but also other STRE-controlled genes are regulated by the growth rate of the cell. The *HXT5* gene, which contains several putative STRE-sequences, is expressed whenever trehalose is accumulated under different growth conditions (Verwaal *et al*, personal communication). The growth-rate dependent behaviour of carbohydrate accumulation generally seems to coincide with the presence of STRE-elements in the promoter of up-regulated genes. Genome-wide analysis of gene expression at different growth rates may reveal a possible general regulatory mechanism in which low growth rates induce expression of STRE-regulated genes.

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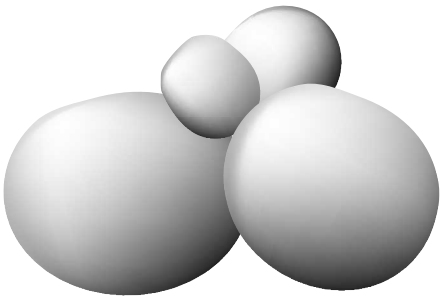
Chapter 4

Function of Trehalose and Glycogen in Cell Cycle Progression and Cell Viability in *Saccharomyces cerevisiae*



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ABSTRACT

Trehalose and glycogen accumulate in *Saccharomyces cerevisiae* when growth conditions deteriorate. It has been suggested that aside from functioning as storage factors and stress protectants, these carbohydrates may be required for cell cycle progression at low growth rates under carbon limitation. By using a mutant unable to synthesize trehalose and glycogen, we have investigated this requirement of trehalose and glycogen under carbon-limited conditions in continuous cultures. Trehalose and glycogen levels increased with decreasing growth rates in the wild-type strain, whereas no trehalose or glycogen was detected in the mutant. However, the mutant was still able to grow and divide at low growth rates with doubling times similar to those for the wild-type strain, indicating that trehalose and glycogen are not essential for cell cycle progression. Nevertheless, upon a slight increase of extracellular carbohydrates, the wild-type strain degraded its reserve carbohydrates and was able to enter a cell division cycle faster than the mutant. In addition, wild-type cells survived much longer than the mutant cells when extracellular carbon was exhausted. Thus, trehalose and glycogen have a dual role under these conditions, serving as storage factors during carbon starvation and providing quickly a higher carbon and ATP flux when conditions improve. Interestingly, the CO_2 production rate and hence the ATP flux were higher in the mutant than in the wild-type strain at low growth rates. The possibility that the mutant strain requires this steady higher glycolytic flux at low growth rates for passage through Start is discussed.

INTRODUCTION

In its natural habitat, the yeast *Saccharomyces cerevisiae* must cope with large fluctuations in the environmental conditions; to do so, it adapts its metabolism to a large variety of external conditions. One such adaptation is to accumulate reserve carbohydrates such as glycogen and trehalose when nutritional conditions deteriorate (Lillie & Pringle, 1980). Initially believed to act as storage factors, trehalose and glycogen were later implicated in other roles as well.

The observation that trehalose and glycogen accumulate not only upon carbon starvation but also under other stress conditions such as nitrogen or sulfur starvation, heat shock, or osmotic stress (Hottiger *et al*., 1987; Lillie & Pringle, 1980; Parrou *et al*., 1997) led to the suggestion that they act as stress protectants rather than as reserve carbohydrates. A role in stress protection has been attributed to trehalose in particular (Wiemken, 1990), since in vitro experiments showed that trehalose protects enzymes

and membranes during dehydration and heat stress (Crowe *et al*., 1984; Hottiger *et al*., 1994) and therefore might act as a stabilizer of cellular structures under stress conditions (Crowe *et al*., 1984). Nevertheless, the relationship between trehalose and glycogen accumulation and stress resistance has remained unclear because mutants in the metabolic pathways of these compounds did not exhibit the expected phenotypes (Nwaka & Holzer, 1998). It was only recently shown that that also in vivo trehalose serves as a protectant during heat shock and prevents denaturation and aggregation of proteins upon heat shock (Singer & Lindquist, 1998).

Another interesting function for trehalose and glycogen came to attention recently: their possible role in cell cycle progression. Studies using synchronized cultures showed that below a particular sugar flux, trehalose and glycogen levels increased during the G_1 phase of the cell cycle and were subsequently degraded upon entry into S phase (Sillje *et al*., 1997). These results have led to the suggestion that trehalose and glycogen may be required under low sugar supply to temporarily increase the sugar flux in order for the cell to complete a cell division cycle (Sillje *et al*., 1997). Interesting in this respect is also the observation that a simultaneous change in trehalose and glycogen levels and budding index can be induced in carbon-limited continuous cultures by transiently increasing the sugar flux (Kuenzi & Fiechter, 1969). The finding that Pho85, a cyclin-dependent kinase, can phosphorylate glycogen synthase isoenzyme 2, resulting in the inactivation of this enzyme, implies a direct link between the cell cycle machinery and trehalose and glycogen metabolism (Huang *et al*., 1996; Timblin *et al*., 1996). In addition to Pho85, protein kinase A (PKA), which is activated by glucose via the RAS/cyclic AMP pathway, plays an important role in trehalose and glycogen metabolism (Thevelein, 1994). However, although this pathway may play an important role in adjusting glycogen and trehalose levels to the external environmental conditions, no cell cycle-dependent changes in PKA activity have been reported.

To further investigate the role of glycogen and trehalose in cell cycle progression at low growth rates under carbon limitation and in the ability to survive starvation, we have made a mutant unable to synthesize these carbohydrates. This was done by deleting the genes *GSY1* (Farkas *et al*., 1990) and *GSY2* (Farkas *et al*., 1991), encoding glycogen synthase isoenzymes 1 and 2, and *TPS1* (Bell *et al*., 1992), encoding trehalose-6-phosphate synthase. By growing this mutant and the isogenic wild-type strain in continuous cultures under sugar limitation conditions, we studied the effects of trehalose and glycogen deficiency on metabolism, cell cycle progression, and survival rate under well-defined conditions.

MATERIALS AND METHODS

Strains and growth conditions.

S. cerevisiae SCU10 (*MAT***a** *SUC2 MAL2-8c MEL tps1*::*TRP1 gsy1*::*LEU2 gsy2*::*URA3*) was constructed from strain CEN-PK113-6B (*MAT***a** *SUC2 MAL2-8c MEL ura3 leu2 trp1*), using deletion plasmids pDH1 (*gsy1*::*LEU2*) and pDH2 (*gsy2*::*URA3*) (Farkas *et al*., 1990; Farkas *et al*., 1991) and a *tps1* deletion plasmid (*tps1*::*TRP1*) (Hohmann *et al*., 1993). As an isogenic wild-type strain, CEN-PK113-7D (*SUC2 MAL2-8c MEL*) was used. Unless otherwise stated, yeast strains were grown at 30°C in yeast nitrogen base without amino acids (YNB; Difco) and with galactose as a carbon source.

Continuous culturing.

Growth in continuous cultures was performed essentially as previously describes (Sillje *et al*., 1996), using a Bioflo III fermentor (New Brunswick) with a 2-liter working volume. Minimal medium for growth in continuous cultures was the same as described previously (Parrou *et al*., 1997). In the feed, galactose was used at a concentration of 10 g l^{-1}. Cells were grown at five different dilution rates (0.033, 0.050, 0.10, 0.15, and 0.20; average mass doubling times of 30, 20, 10, 7.5, and 5 h, respectively). From every steady state, samples were taken for 3 successive days in duplicate. Levels of CO_2 production and O_2 consumption were measured on-line by connection of the headspace of the fermentor to a URAS3G carbon dioxide analyzer and a MAGNOS4G oxygen analyzer (Hartmann & Braun). Since under all conditions the respiration quotient (carbon dioxide rate divided by oxygen consumption rate) remained constant, and thus metabolism was totally respiratory, the ATP flux could be calculated by multiplying the oxygen consumption rate by 6.

Analysis of cell sizes and numbers.

Cell sizes and cell numbers were determined with an electronic particle counter (Coulter Counter). Cell sizes were calculated by calibration with latex beads of known sizes. Dry weights were determined by spinning down 20 ml of culture volume in duplicate and washing the cells with an equal amount of water. Cell pellets were subsequently transferred into preweight bottles and dried for at least 12 h at 95°C. The increase in bottle weight was multiplied by 50 to give the dry weight per liter of culture volume.

Determination of galactose consumption rates.

Samples of 2 ml for the determination of residual galactose concentrations

were taken and directly filtered through a 0.22-µm-pore-size filter. The supernatant fractions were stored at 20°C until galactose determination. Galactose concentrations were measured as described previously (Sillje *et al*., 1997). The galactose consumption rate was calculated by subtracting the amount of residual galactose from the amount of galactose added per time unit divided by cell number.

Determination of trehalose and glycogen levels.

Samples of 2 ml were centrifuged for 30 s at 4,000 rpm. Medium was discarded, and cells were suspended in ice-cold water and centrifuged again. The cell pellets obtained were quickly frozen into liquid nitrogen and stored at 80°C until further processing. Trehalose and glycogen were extracted and measured essentially as described previously (Sillje *et al*., 1997).

RESULTS

Construction and verification of a glycogen and trehalose synthesis-deficient strain.

To investigate the roles of trehalose and glycogen under different growth conditions, we constructed mutant strain SCU10, in which the *TPS1* (trehalose-6-phosphate synthase), *GSY1* (glycogen synthase 1), and *GSY2* (glycogen synthase 2) genes were deleted (see Materials and Methods). Since a deletion in *tps1* is lethal when such a strain is grown in glucose-containing medium, due to uncontrolled glucose uptake (Thevelein & Hohmann, 1995), all experiments were performed with galactose as the carbon source. In strain SCU10, the trehalose and glycogen levels were all below the detection level, indicating that this strain could not synthesize these carbohydrates (figure1). We observed no differences in growth rate (mean ± standard error of the mean [SEM]) between strain SCU10 and the isogenic wild-type strain CEN-PK113-7D after growth on 2% galactose.

Continuous culturing.

Since trehalose and glycogen are accumulated at low growth rates and especially under carbon-limited conditions, the wild-type and mutant strains were cultivated in galactose-limited continuous cultures, in which the growth rate can be accurately regulated by controlling the feed rate. This enabled us to analyze physiological parameters such as dry weight, sugar consumption rate, CO_2 production rate, and O_2 consumption rate at different growth rates under well-defined conditions.

By changing the dilution rate in these cultures between 0.20 and 0.033 h^1, the biomass doubling time was varied between 5 and 30 h. In the wild-type strain, the trehalose and glycogen levels increased from 0 to 6.2 fmol of glucose $cell^{-1}$ and from 2 to 11 fmol of glucose/cell, respectively, with decreasing doubling time from 5 to 30 h (figure 1). We detected no trehalose and glycogen in strain SCU10.

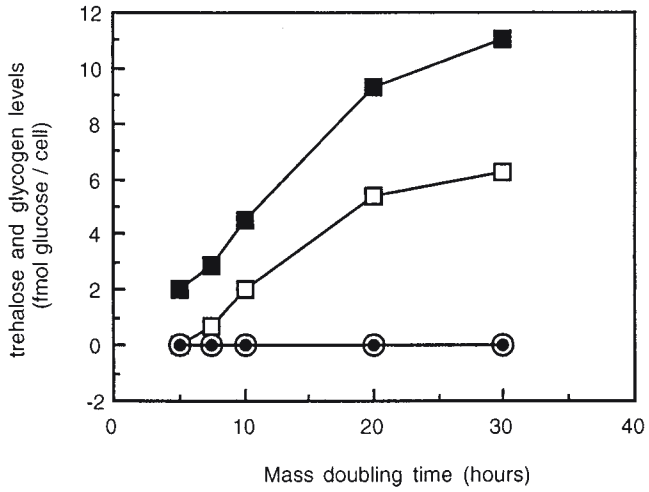

Figure 1: **Trehalose and glycogen levels in continuous cultures of *S. cerevisiae* CEN-PK113-7D (wild type) and SCU10.** Galactose-limited continuous cultures of strains CEN-PK113-7D and SCU10 were grown at different dilution rates, yielding different doubling times. At every steady state, samples were taken for 3 successive days and analyzed for the trehalose and glycogen contents. Therefore, at every doubling time trehalose and glycogen levels were measured in triplicate. SEMs were always <0.29 and <0.75 fmol of glucose $cell^{-1}$ h^{-1} for trehalose and glycogen, respectively, (□) trehalose, CEN-PK113-7D; (■) glycogen, CEN-PK113-7D; (❍) trehalose, SCU10; (●) glycogen SCU10.

Cell numbers and biomass concentration.

To see if the mutant strain was affected in its ability to grow under these conditions, we measured cell numbers and biomass concentrations in the cultures. We found that cell numbers were the same in the wild-type and mutant strains and decreased from approximately 470 x 10^6 to 230 x 10^6 cells ml^{-1} with increasing doubling time from 7.5 to 30 h (figure 2A). Thus, mutant strain SCU10 can grow under all conditions tested with doubling times similar to those for the wild-type strain, and hence glycogen and trehalose accumulation is not essential at low growth rates. However, we noted a clear decrease in dry weight levels in strain SCU10, ranging from

4.5 to 2.7 g l^{-1} with increasing doubling time (from 0.20 to 0.033 h^{-1}), whereas the dry weight of the wild-type culture remained around 4.5 g l^{-1} under these conditions (figure 2B). This resulted in an increase in dry weight per cell in the wild-type strain ranging from 10.6 to 17.4 pg $cell^{-1}$ with decreasing growth rates, whereas the level remained almost constant in the mutant strain (figure 2C). From comparison of these data with the trehalose and glycogen levels shown in figure 1 it can be calculated that the increase in dry weight in the wild-type cells is due mainly to the increase in trehalose and glycogen levels at low growth rates (figure 2C). Thus, the decrease in cell numbers in the wild-type culture with decreasing growth rate can be explained by the fact that this strain, unlike the mutant strain, accumulates storage carbohydrates, thereby leaving less sugar available for the synthesis of cell components.

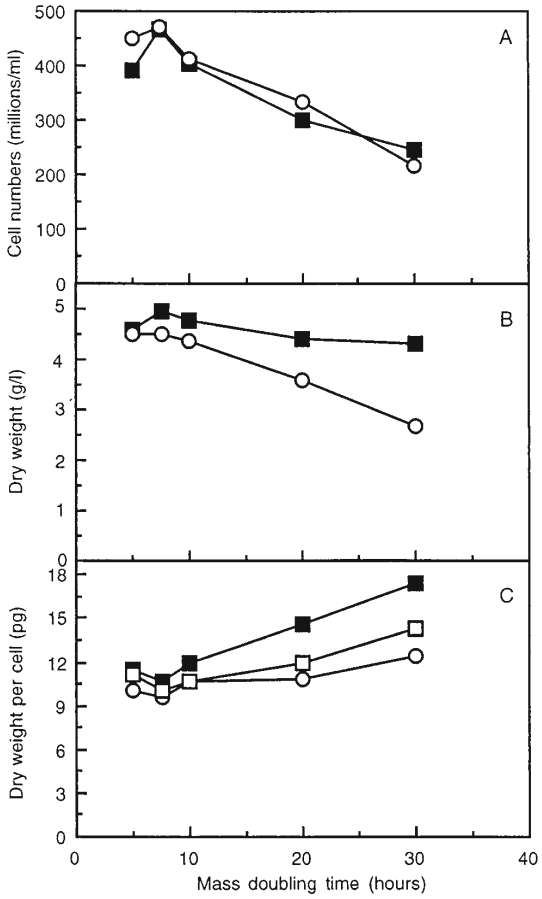

Figure 2: **Biomass concentrations in continuous cultures of strains CEN-PK113-7D (wild type) and SCU10.** Galactose-limited continuous cultures of strain CEN-PK113-7D (■) and SCU10 (❍) were grown at different dilution rates, yielding different doubling times. At every steady state, samples were taken for 3 successive days for analyses of cell numbers (A) and dry weights (B). SEMs for dry weights and cell numbers were always <0.15 g/liter and <36 x 10^6 cells/ml, respectively. By dividing the dry weight levels by the cell numbers at every doubling time, dry weights per cell were calculated (C). For the wild-type strain CEN-PK113-7D, also the amounts of trehalose and glycogen as shown in figure 3 were subtracted from the biomass per cell (□).

Carbon flux at different growth rates.

Since strain SCU10 does not accumulate reserve carbohydrates, while cell numbers decreased as in the wild-type culture, part of the sugar was apparently lost. The sugar consumption rates of the wild-type and mutant strains decreased similarly with increasing doubling time, indicating no difference in sugar consumption between the two strains (figure 3A). Therefore, at low growth rates some of the carbon in the

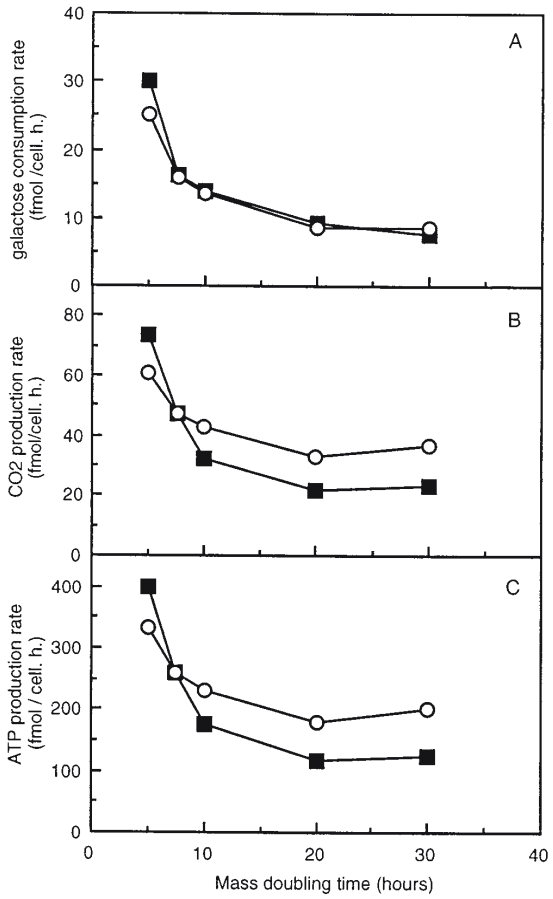

Figure 3: **Carbon fluxes in continuous cultures of strain CEN-PK113-7D (wild type) and SCU10.** Galactose-limited continuous cultures of strain CEN-PK113-7D (■) and SCU10 (❍) were grown at different dilution rates, yielding different doubling times. At every steady state, samples were taken for 3 successive days for analyses of residual galactose, from which the consumption rate was calculated (see Materials and Methods) (A). CO_2 production was measured online (SEM < 3.6 fmol $cell^{-1}$ h^{-1} (B). ATP production (C) was calculated from the CO_2 production rate.

mutant must flow in directions other than biomass. Analyses of CO_2 production rates showed that at high growth rates the wild-type and mutant strains behaved identically, but at low growth rates (doubling times of between 20 and 30 h) the mutant produced much more CO_2 (36 fmol $cell^{-1}$ h^{-1}) than the wild type (24 fmol $cell^{-1}$ h^{-1}) (figure 3B). Thus,

the sugar which is stored in the wild type as trehalose and glycogen is oxidized in the mutant. Because under all conditions tested here metabolism was strictly aerobic and the respiration quotient was always about 1.2 (data not shown), this finding means that at low growth rates the ATP flux is much higher in strain SCU10 than in the wild-type strain. The ATP flux (figure 3C) is about 1.6 times higher in the mutant than in the wild type at low growth rates. The reason for this difference is not clear. However, since it has been shown that at low growth rates under carbon limitation conditions wild-type strains degrade trehalose and glycogen upon cell cycle progression (Sillje *et al*., 1997), the increase in carbon and ATP flux may be important for traversing the cell cycle. Since it cannot increase its carbon flux temporarily, it is possible that strain SCU10 must keep its energy flux higher throughout the cell cycle at low growth rates.

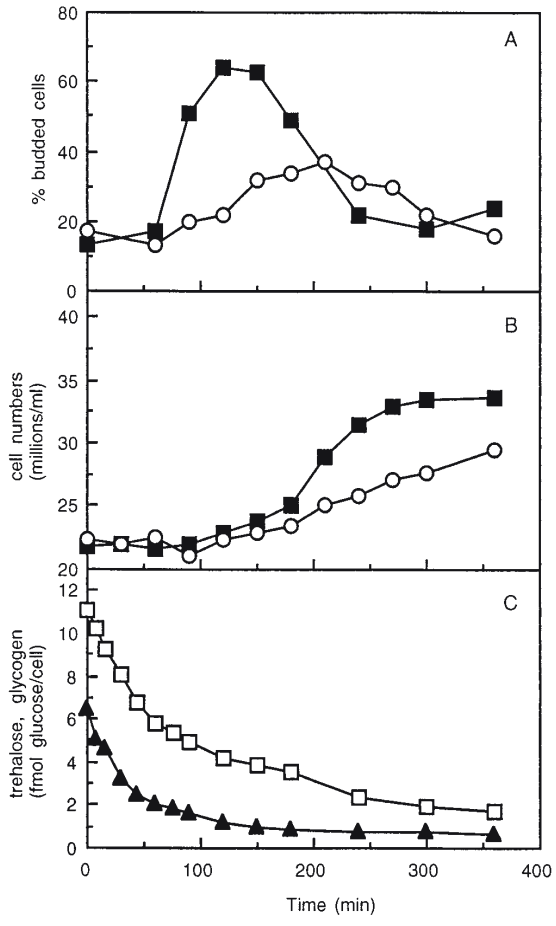

Figure 4: **Reaction with respect to cell cycle progression after an increase in extracellular sugar concentration.** From galactose-limited continuous cultures growing with average doubling times of 30 h, a sample was taken and diluted 10 times in YNB-2 mM galactose. At different time points, percentages of budded cells (A) and cell numbers (B) and trehalose and glycogen levels in strain CEN-PK113-7D (C) were determined.(■), wild-type CEN-PK113-7D;(❍), mutant SCU10;(▲), trehalose;(□), glycogen.

Cell cycle progression.

To demonstrate that the wild-type strain has an advantage over strain SCU10 with respect to the rate of cell cycle progression, cells were taken from the fermentor (at doubling times of 30 h) and diluted 10-fold in medium with 2 mM galactose. The residual concentration in the fermentor is in both cases 0.4 mM, which results in a fivefold increase in galactose concentration. Most wild-type cells were directly triggered to enter S phase, as shown by the increase in percentage of budded cells from 13 to 64% 120 min after inoculation in 2 mM galactose (figure 4A). In strain SCU10, this increase in percentage of budded cells was much slower and the maximal level was about 37%. As expected, also the cell numbers increased faster in the wild-type cells and, upon consumption of all galactose, were higher for this strain than for strain SCU10 (figure 4B). Immediately after galactose addition, wild-type cells started to degrade trehalose and glycogen, resulting in a carbon and ATP flux higher than that for strain SCU10 (figure 4C). Thus, this degradation of trehalose and glycogen may give the wild-type strain a strong advantage.

Survival rate.

Although it is likely that trehalose and glycogen function as reserve carbohydrates upon carbon starvation and decreases in their levels have been observed upon prolonged starvation (Lillie & Pringle, 1980), a direct link has never been shown. Therefore, we inoculated wild-type and SCU10 cells from continuous cultures growing with doubling times of 30 h (high trehalose and glycogen levels in the wild type) into minimal medium without carbon source and monitored survival over time (figure 5). No change in the amounts of viable cells was observed in the wild-type culture for at least 10 days. The SCU10 cells, however, started to lose viability after approximately 24 h, and after about 192 h only 1% of the cells were still viable. Similar experiments with mutants unable to synthesize only trehalose or glycogen showed no decreased cell viability, indicating that both trehalose and glycogen function as reserve carbohydrates. In addition, we observed that during starvation, the wild-type strain consumes both trehalose and glycogen (data not shown). These results show that both trehalose and glycogen function as reserve carbohydrates under carbon starvation conditions.

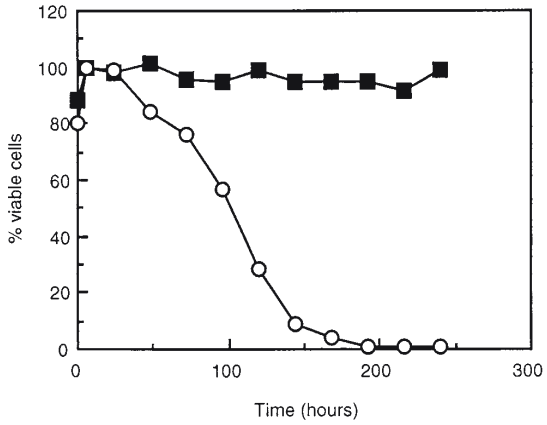

Figure 5: **Survival of strains CEN-PK113-7D (wild type; ■) and SCU10 (mutant; ❍) on medium without carbon source.** From galactose-limited continuous cultures growing with average doubling times of 30 h, a sample was taken and diluted 10 times in YNB without carbon source. At different time points, cells were plated onto plates containing 2% galactose, and the number of colonies were counted after 3 days of incubation at 30°C. The maximal number of colonies obtained in each culture was set at 100% viability. (Maximal numbers of cells were obtained about 5 to 6 h after incubation, probably because of the presence of cells which had to finish their cell cycle)

DISCUSSION

Recently we showed that at low growth rates under carbon limitation, trehalose and glycogen accumulate during the G_1 phase of the cell cycle and are subsequently degraded again upon cell cycle progression (Sillje *et al*., 1997). This finding suggested that trehalose and glycogen may be essential to generate a carbon flux sufficient for rapid progression through the cell division cycle under poor growth conditions. Here we show, however, that a mutant unable to synthesize these carbohydrates can still grow at low rates under carbon limitation. Thus, trehalose and glycogen are not required for cell cycle progression under such conditions. Interestingly, however, the mutant had a much higher ATP flux under those conditions compared to the wild-type strain, which it maintained by completely oxidizing the amount of sugar normally used for synthesizing trehalose and glycogen. Thus, instead of making more biomass under those conditions, the mutant strain seems to use this surplus of sugar by generating a higher ATP flux. Therefore, it is tempting to suggest that whereas the wild type can momentarily increase its flux by the degradation of trehalose and glycogen upon cell cycle progression (Sillje *et al*., 1997), the mutant must keep its flux

continuously higher in order to go through a cell division cycle.

Why then does the wild-type strain accumulate trehalose and glycogen under these conditions and degrade it again upon entry into a cell division round if doing so provides no obvious advantage? The answer to this question becomes clear when the sugar flux is temporarily increased, in which case the wild-type strain can go through a cell division round much faster than the mutant strain by degrading its reserve carbohydrates and hence increasing its glycolytic flux. Also the opposite condition, namely, a further drop in sugar flux, gives the wild-type strain an advantage, since under carbon starvation it survives much better than the mutant strain. Thus, yeast cells accumulate these carbohydrates under such conditions so as to be well prepared to either survive a long period under worse conditions or go quickly through a cell division round when conditions improve. Since under natural conditions these kinds of changes are quite normal, cells that can synthesize trehalose and glycogen will have had a strong evolutionary advantage over cells that cannot.

How trehalose and glycogen metabolism is regulated under these conditions is not known. As mentioned, a good candidate is the cyclin-dependent Pho85 protein kinase, which like the Cdc28 cyclin-dependent kinase is involved in the regulation of cell cycle progression (Espinoza *et al*., 1994; Measday *et al*., 1994). Pho85 can, in conjunction with the cyclins Pcl8 and Pcl10, phosphorylate glycogen synthase 2 kinase, resulting in the down regulation of glycogen synthase 2 activity (Huang *et al*., 1996; Huang *et al*., 1998; Timblin *et al*., 1996). It will therefore be interesting to determine whether Pho85 regulates glycogen and trehalose metabolism in a cell cycle- and nutrition-dependent way. Nevertheless, other regulators such as PKA may also play a role in this regulation.
In conclusion, it appears that trehalose and glycogen may have a dual function under sugar limitation conditions. Upon a decrease in growth rate, cells start to accumulate glycogen and trehalose, which can then be used for maintenance if extracellular carbohydrates become exhausted or used to quickly go through a cell division round when conditions improve.

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Addendum chapter 4:

INTRODUCTION

At low growth rates, trehalose and glycogen are accumulated during the G_1 phase and degraded prior to S phase entry (chapter 3; Sillje *et al*., 1997). The degradation of these carbohydrates generates an increase in the ATP flux, which may be required for progression through the cell cycle. However, mutants unable to accumulate trehalose and glycogen are still able to grow at low growth rates. One of the remaining questions is how cell cycle progression is regulated at low galactose consumption rates in the carbohydrate-deficient mutants. Whereas wild-type cells spend high amounts of energy in the accumulation of trehalose and glycogen, the mutant deficient in carbohydrate accumulation uses its energy to produce a high ATP flux when forced to grow at low growth rates. Here, we show that the mutant deleted for both trehalose and glycogen synthase is able to progress through the cell cycle at a faster rate than wild-type cells at low sugar consumption. Thus, the accumulation of trehalose and glycogen accumulation negatively influences cell cycle progression at low carbon fluxes.

As a possible function in cell cycle progression, it was shown that the presence of both trehalose and glycogen induces cell cycle progression in response to an increase in extracellular sugar and increases viability of the cells. Here, the separate roles of trehalose and glycogen accumulation in cell viability are described.

MATERIALS AND METHODS

Yeast strains and plasmids

The haploid strain CEN-PK 113-7D (*MAT***a** *SUC2 MAL2-8c MEL*) was used as wild-type strain in all experiments. The *tps1* deletion strain was constructed from strain CEN-PK 113-3C (*MAT***a** *SUC2 MAL2-8c MEL trp1*), using a *tps1* deletion plasmid (*tps1::TRP1*). The *gsy1, gsy2* deletion strain was constructed from strain CEN-PK 102-3A (*MAT***a** *SUC2 MAL2-8c MEL ura3 leu2*), using deletion plasmids pDH1 (*gsy1::LEU2*) and pDH2 (*gsy2::URA3*) . The *tps1*, *gsy1*, *gsy2* triple-deletion mutant (strain SCU10) was constructed from strain CEN-PK 113-6B (*MAT***a** *SUC2 MAL2-8c MEL ura3 leu2 trp1*) by using the three deletion plasmids described above.

Growth conditions

All experiments were performed at 30^0C in yeast nitrogen base without amino-acids (6.7 g l^{-1} YNB, Difco) with galactose as carbon source. Synchronous fed-batch cultures were performed in YNB medium with a constant residual galactose concentration of 0.15 mM at a cell density of 1-2 x 10^7 cells ml^{-1}. Galactose was continuously added at rates ranging from 12 fmol $cell^{-1}$ h^{-1} to 20 fmol $cell^{-1}$ h^{-1}. The cell number and the external galactose concentration were monitored throughout the growth. Galactose consumption rates were determined as described previously (Sillje *et al.*, 1997).

Cell synchronization

Centrifugal elutriation was performed essentially as described previously (Woldringh *et al*., 1993), with some modifications. The yeast strains were grown exponentially in YNB medium containing 1% galactose at 30^0C and 2 x10^{10} cells were loaded in a 40 ml chamber of a Beckman J-6MI centrifuge (JE-5.0 rotor) at 30 ^{0}C and 2000 rpm. Cells were cultivated in the elutriator chamber on YNB medium containing 1% galactose. Small cells were washed out at a flow rate of 45 ml min^{-1}. and newborn daughter cells were collected on ice. The cell size was monitored with a Coulter Multisizer II and the flow rate of the elutriation was adapted to remain a constant cell size.

Analysis of sample parameters.

Cell sizes and cell numbers were determined with an electrical particle counter (Coulter Multisizer II). Cell sizes were calculated by calibration with latex beads of known size. Budding percentages were determined by counting at least 200 cells microscopically.

Determination of trehalose and glycogen levels.

Samples of 25 ml were centrifuged for 30 s at 4000 rpm (Hettich centrifuge). The cells were washed in ice-cold water and trehalose and glycogen was extracted from the pellet as described previously (Sillje *et al.*, 1997).

RESULTS AND DISCUSSION

Trehalose and glycogen accumulation causes elongation of the G_1 phase in fed-batch cultures.

In carbon-limited fed-batch cultures, the accumulation of reserve carbohydrates coincides with a substantial increase in the G_1 phase duration (Chapter 3, figure 1). As a substantial part of the available energy is used in the accumulation of trehalose and glycogen in wild-type cells, accumulation of reserve carbohydrates may cause the elongation of the G_1 phase. The effect of trehalose and glycogen accumulation on cell cycle progression was studied in synchronous fed-batch cultures. Wild-type cells (strain CEN-PK113-7D) and cells deleted for trehalose and glycogen synthetase (SCU10) were synchronized by centrifugal elutriation in early G_1 phase and grown in fed-batch culture at different galactose consumption rates.

As shown in figure 1, a biphasic correlation was observed between the galactose flux and the amount of carbohydrates accumulated in wild-type cells . At galactose consumption rates of 20 fmol galactose cell^{-1} h^{-1} and higher, only low amounts

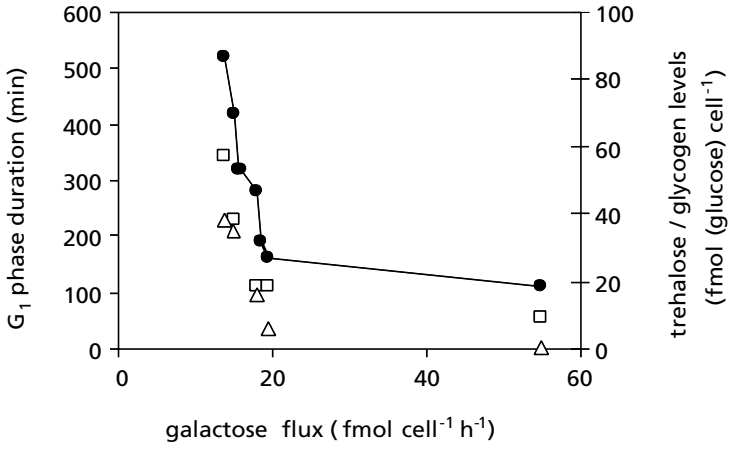

Figure 1: **Correlation between the duration of the G_1 phase, trehalose and glycogen levels and the galactose consumption rate of the cell.**
Cells were synchronised by elutriation and grown on minimal medium containing either 1% galactose or under galactose limitation at different galactose consumption rates. The maximal trehalose and glycogen level at the end of the G_1 phase was determined, whereas the G_1 phase duration was defined as the time from inoculation until 50% budding was reached. (●) G_1 phase duration (min.), (□) glycogen (fmol (glucose) cell^{-1}), (Δ) trehalose (fmol (glucose) cell^{-1}).

of trehalose and glycogen were accumulated. As the galactose consumption rate decreased from 20 to 14 fmol galactose cell^{-1} h^{-1}, trehalose and glycogen accumulation rapidly increased to levels of 38 and 57 fmol (glucose) cell^{-1} respectively at 14 fmol galactose cell^{-1} h^{-1}.

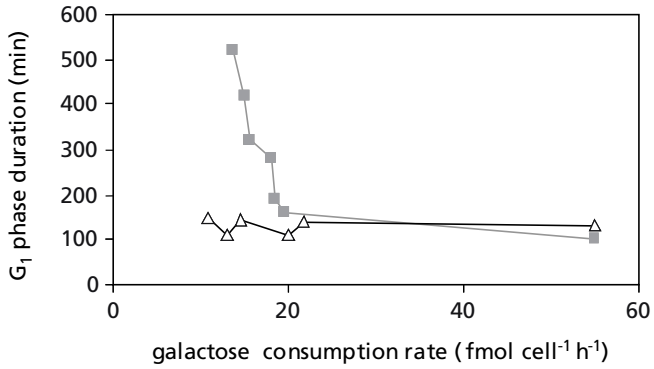

Figure 2: **Cell-cycle progression through the G_1 phase of *S. cerevisiae* in wild-type cells and strain SCU10, deficient in trehalose and glycogen accumulation.**
Cells were synchronised by centrifugal elutriation and grown in minimal medium containing 1% galactose or in fed-batch cultures at different galactose consumption rates. The duration of the G_1 phase was defined as the time from inoculation until 50% budding was reached. (■) wild-type strain CEN-PK113-7D, (Δ) strain SCU10.

At high galactose consumption rates, both wild-type and SCU10 strain have a short G_1 phase of 110 and 130 minutes respectively at 55 fmol cell^{-1} h^{-1} (figure 2). As reserve carbohydrates become accumulated, the G_1 phase duration of wild-type cells rapidly increases from 160 minutes at 20 fmol galactose cell^{-1} h^{-1} to 520 minutes at 14 fmol galactose cell^{-1} h^{-1}. Surprisingly, the G_1 phase duration of the SCU10 deletion strain remained short at 120 minutes when grown at galactose consumption rates of 20 fmol galactose cell^{-1} h^{-1} and lower. Furthermore, cells completely finished their cell cycle under these growth conditions, as newly formed cells were observed after 200 minutes of growth. Thus, the SCU10 deletion strain is able to progress through the cell cycle at a faster rate than the wild-type strain under low galactose consumption rates. These results indicate that accumulation of trehalose and glycogen itself causes elongation of the G_1 phase under low carbon supply.

The accumulation of trehalose and glycogen requires a substantial amount of the available nutrients, which could decrease the amount of energy available for cell cycle progression. Thus, the deletion mutant could use more of the available energy for progression through the cell cycle. The pathway linking the amount of available energy to progression through the cell cycle remains to be identified. Possibly, the cAMP/PKA pathway is involved in this regulation, as this pathway is upregulated at the time of carbohydrate degradation and is required for cell cycle progression (Hubler *et al*., 1993; Timblin *et al*., 1996).

Both trehalose and glycogen function as reserve carbohydrates.

The effects of trehalose and glycogen accumulation on survival of cells was studied by comparing survival of wild-type cells with cells deleted for trehalose synthase (*tps1*), glycogen synthase (*gsy1 gsy2*) and both synthases (*tps1 gsy1 gsy2*; strain SCU10). Cells were grown overnight on minimal medium containing 2% galactose until stationary cells were obtained. After inoculated in fresh medium lacking a carbon source, the trehalose and glycogen content were measured in time and the survival rate was determined.

Stationary phase wild-type cells have accumulated trehalose and glycogen at levels of 33 and 66 fmol (glucose) cell^{-1} respectively (figure 3A). After 8 days of starvation, trehalose and glycogen levels were decreased to 0.4 and 12 fmol (glucose) cell^{-1} respectively, whereas the cells remained fully viable. These results indicate that both trehalose and glycogen are metabolized during starvation and wild-type cells remain viable for at least 8 days. The SCU10 strain is unable to accumulate carbohydrates and viability is decreased to 42% after 8 days of starvation (figure 3D). Thus, when both trehalose and glycogen are present in the cell, the viability of cells is increased.

To discriminate between the ability of trehalose and glycogen to increase cell survival upon carbon starvation, mutants in either trehalose or glycogen synthetase were studied for viability. The *tps1* mutant showed increased glycogen accumulation in the stationary phase when compared to the wild type, as 135 fmol (glucose) cell^{-1} glycogen was accumulated (figure 3B). After 8 days of starvation, 50% of the glycogen was metabolized and more than 90% of the cells remained viable. These results indicate that glycogen accumulation and metabolization upon carbon starvation increases cell viability. The *gsy1 gsy2* mutant had similar trehalose levels accumulated as wild-type cells, at 29 fmol (glucose) cell^{-1} (figure 3C). Upon starvation for 8 days, 80% of the trehalose was metabolized while the cells remained viable. Thus, trehalose accumulation and metabolization increases cell viability upon carbon starvation. These

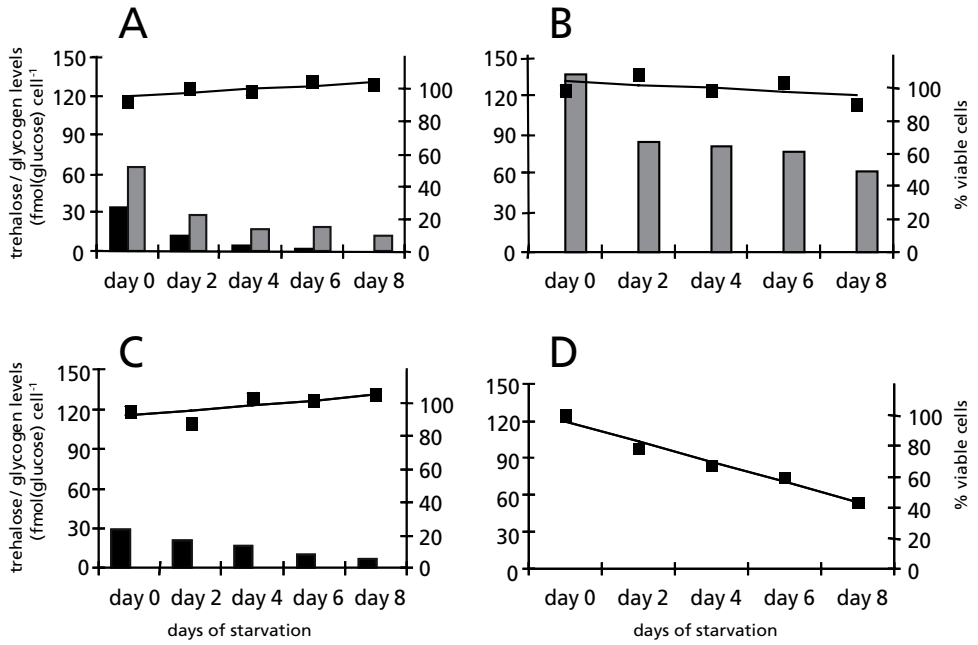

Figure 3: **Carbon-starvation of the wild-type strain and strains deficient in trehalose and glycogen synthesis.**
Cells were grown overnight in minimal medium containing 2% galactose until stationary phase. Subsequently, the cells were inoculated in minimal medium without carbon source. At different time points, the trehalose (■) and glycogen (■) levels were determined. The viability (—■—) of the cells was assayed by plating cells onto plates containing YNB medium and 2% galactose. Colonies were counted after 3 days and compared with the original amount of cells. (A) wild-type strain CEN-PK113-7D, (B) *tps1* strain, (C) *gsy1 gsy2* strain, (D) strain SCU10.

results suggest that trehalose and glycogen function both as reserve carbohydrates under carbon starvation.

Summarising, the ability to accumulate trehalose and glycogen results in a delay in cell cycle progression, when cells are grown at low carbon fluxes. Since yeast cells have to deal with large fluctuations in environmental conditions in their natural habitat, they are able to overcome prolonged periods of nutrient starvation and can remain viable under these conditions. Our results show that the survival rate of cells is highly increased by both trehalose and glycogen levels under carbon starvation. These beneficial effects of trehalose and glycogen accumulation seem to counterbalance the negative effects in cell cycle progression.

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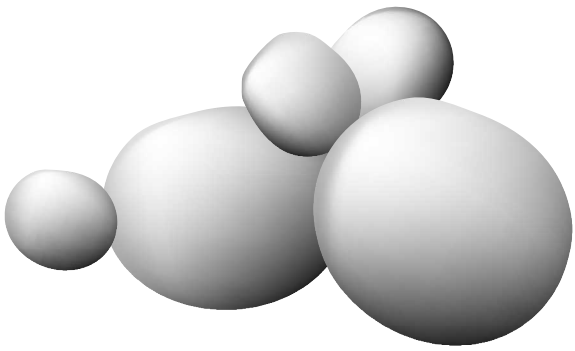
Chapter 5

General Discussion



General Discussion

The duration of the G_1 phase of the cell cycle increases when the nutrient availability is decreased. By using galactose-limited fed-batch cultures, we showed that the duration of the G_1 phase is rapidly increased at galactose consumption rates of 18 fmol $cell^{-1}$ h^{-1} and lower. Under these growth conditions, the cell accumulates trehalose and glycogen during the G_1 phase of the cell cycle. In chapter 2, a signalling pathway is described that is involved in triggering cell cycle progression in response to the nutrient availability. It was shown that phosphorylation of the MAP kinase Slt2 results in a reduction in the G_1 phase duration. The Slt2 protein is part of the PKC signalling pathway that can phosphorylate and activate the Swi4/Swi6 (SBF) complex *in vitro* (Gustin *et al*., 1998; Lee *et al*., 1993; Madden *et al*., 1997). The SBF complex is present in the nucleus throughout the G_1 phase and remains in an inactive form until the end of the G_1 phase (Baetz & Andrews, 1999; Harrington & Andrews, 1996; Taba *et al*., 1991). When the Swi6 protein enters the nucleus late in the M phase, it allows binding of Swi4 to the DNA (Baetz and Andrews, 1999). At the end of the G_1 phase, the Swi6 protein is phosphorylated at serine 160 and diffuses into the cytoplasm (Sidorova *et al*., 1995). Therefore, phosphorylation of Swi6 by Slt2 results in the localization of Swi6 towards the cytoplasm, whereas Slt2-dependent phosphorylation of Swi4 may be involved in inducing transcription in late G_1 (Madden *et al.*, 1997). As the SBF complex is activated in the nucleus, it is likely that the MAP kinase Slt2 is translocated towards the nucleus upon activation, as was described for higher eukaryotic cells (Hulleman *et al*., 1999). Although no relocalization of Slt2 towards the nucleus has been described, another MAP kinase, Hog1, does enter the nucleus upon activation (Ferrigno *et al*., 1998; Van Wuytswinkel *et al*., 2000). Thus, MAP kinases may transduce signals from the cell surface towards the nucleus by translocation.

In our experiments, Slt2 was only activated at high sugar consumption rates, whereas Slt2 phosphorylation was not induced at low consumption rates. At high sugar consumption rates, the cell size increases more rapidly than at lower consumption rates. Therefore, Slt2 may induce cell cycle progression in response to the growth rate of the cell in wild-type conditions. Indeed, the cell size is reduced in cultures with an increase in Slt2 phosphorylation (data not shown). In agreement with this, deletion of *SLT2* results in an increase in the cell size (Mazzoni *et al*., 1993; data not shown). Thus, nutrients may control G_1 phase progression by induction of Slt2 phosphorylation upon cell growth.

The amount of phosphorylated Slt2 seems to depend on the nutrient flux in the cell (chapter 2; Zarzov *et al.*, 1996). One of the main cell cycle regulators responding to

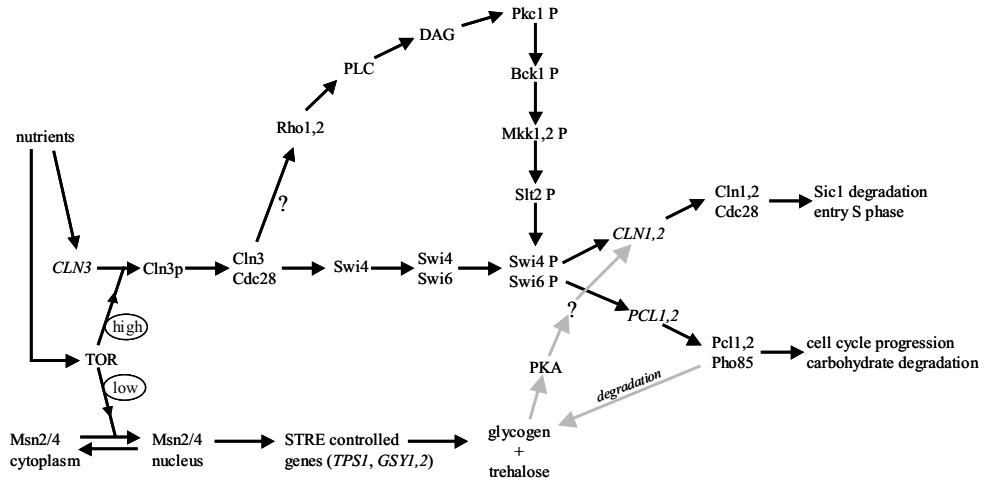


Figure 1: **Model for regulation of G_1 phase progression in *S.cerevisiae***. High nutrient availability results in induction of cell cycle progression by phosphorylation of the Swi4/Swi6 (SBF) complex by Slt2. Low nutrient fluxes result in the accumulation of trehalose and glycogen during the G_1 phase and a strong increase in the cell cycle duration. The SBF complex remains unphosphorylated under these conditions. For more details see text.

the nutrient availability is the G_1 cyclin Cln3 (Hubler *et al*., 1993; Parviz *et al*., 1998; Parviz & Heideman, 1998). Upon overexpression of *CLN3* at low galactose fluxes, cells progress through the G_1 phase more rapidly than wild-type cells and phosphorylation of Slt2 is strongly increased (chapter 2). This indicates that Slt2 phosphorylation functions downstream of Cln3 in progression through the G_1 phase (figure 1). In agreement with this, previous studies have shown that Cln3/CDK activity is involved in activation of the SBF complex, whereas no direct activation of SBF by Cln3/Cdc28 kinase activity has been detected (Dirick *et al*., 1995; Koch *et al*., 1996; Stuart & Wittenberg, 1995). The pathway by which the Cln3/Cdc28 kinase activates Slt2 remains to be identified, but is likely to involve the PKC MAP kinase pathway. During the G_1 phase, an increase in diacylglycerol (DAG) levels was observed that was induced by Cdc28 activity at Start (Marini *et al*., 1996). As the activity of Pkc1 is increased upon addition of DAG *in vivo*, the PKC pathway could well function downstream of Cln3/Cdc28 activity at Start (Ogita *et al*., 1990; figure 1). Nevertheless, several attempts to show this interaction *in vivo* remained unsuccesfull, but this is probably due to experimental problems (discussed in Gustin *et al*., 1998).

Other proteins may also be involved in activation of the PKC pathway at Start. The Pkc1 protein can be activated through a pathway composed of the proteins TOR2 and the Rho-type GTPase Rho1 (Helliwell *et al*. [B], 1998; Kamada *et al*., 1996; Nwaka *et al*., 1995; Schmidt *et al*., 1997). The Rho1 protein is required for Pkc1-dependent cell cycle progression and polarised growth (Andrews & Stark, 2000; Delley & Hall, 1999; Helliwell *et al*., 1998 [A]; Helliwell *et al.*, 1998 [B]). The activity of the GTPase Rho1 is under control of the GDP/GTP exchange factor Rom2, which functions downstream of the phosphatidylinositol kinase homolog TOR2 (Schmidt *et al.*, 1997). Rom2 can only interact with GDP-bound Rho1, whereas the GTP-bound form of Rho1 can interact with Pkc1 (Nonaka *et al*., 1995; Philip & Levin, 2001). Interestingly, the active form of Rho1 is required for proper localization of Pkc1 to the pre-bud site (Andrews & Stark, 2000). The Rho1 protein was localized to different compartments of the cell, including secretory vescicles, whereas Pkc1 is localised to the site of polarised growth (Andrews & Stark, 2000). Therefore, the GTP-bound Rho1 may bind to Pkc1 and subsequently translocate Pkc1 to the site of budding (Qadota *et al*., 1996; Yamochi *et al*., 1994). These results indicate that Pkc1 functions downstream of the TOR/Rho1 pathway in the G_1 phase of the cell cycle. In agrement with this, G_1 arrest by a *tor2* mutation can be overcome by overexpression of *RHO1*, *RHO2, ROM2*, *SLT2* or *BCK1-2*, an active allele of Bck1 of the PKC pathway (Helliwell *et al.*, 1998 [A]; Schmidt *et al.*, 1997). Moreover, overexpression of *SLT2* resulted in recovery from the actin polymerization defect of the *tor2* strain (Helliwell *et al.*, 1998 [B]). Therefore, also the downstream targets of Pkc1 in the PKC signalling pathway function downstream of TOR2 in regulating polarized growth in the G_1 phase (figure 1). Possibly, also the phosphatidylinositol pathway is involved in regulating G_1 phase progression, as G_1 arrest by *tor2* mutation is overcome by overexpression of *PLC1* (Helliwell, et al., 1998 [A]). The *PLC1* gene encodes phosphatidylinositol (PI)-dependent phospholipase C and functions in the hydrolysis of PI and the formation of diacylglycerol (Flick & Thorner, 1993). As described above, diacylglycerol levels are increased during the G_1 phase and can activate Pkc1 in vitro (Ogita *et al.*, 1990). The Plc1 protein may therefore function as a last step in activating Pkc1 downstream of TOR2 (figure 1). As for Plc1 and Rho1, Cln3 also functions downstream of the TOR pathway and upstream of the PKC signalling pathway (Barbet *et al*., 1996; chapter 2). This may imply that Cln3/Cdc28 activity is an intermediate of the Rho1/Plc1 pathway or a cell-cycle dependent activator of this pathway that controls the timely activation of actin polarisation during the G_1 phase of cell cycle (figure 1).

Interestingly, expression of the *CLN1* and *CLN2* genes peaks at the end of the G_1 independent of the G_1 phase duration (Sillje *et al*., 1997). The peak in transcription of cells with a short G_1 phase is induced by phosphorylation of the SBF complex by the

MAP kinase Slt2. However, Slt2 does not seem to be involved in the induction of cyclin expression during long G_1 phases, as no phosphorylated Slt2 was detected during the G_1 phase of these cells. In agreement with this, deletion of *SLT2* resulted in a delay in progression through the G_1 phase, although these cells were still able to grow and divide. As *slt2* cells do not show phosphorylated Swi4 and Swi6 (Madden *et al.*, 1997), this indicates that the (unphosphorylated) SBF complex may induce transcription of the late-G_1 cyclins at a low rate. Otherwise, another transcription factor could be involved in activating cyclin transcription in late G_1, like the Mbp1/Swi6 transcription factor that also regulates timely transcription of genes during the G_1 phase (Koch *et al*., 1993).

One of the main regulators of the level of Cln3 in the cell is the activity of the TOR kinase pathway (Barbet *et al.*, 1996). Upon inhibition of TOR by rapamycin, translation of Cln3 is inhibited and cells arrest in the G_1 phase (Barbet *et al.*, 1996; Helliwell *et al.*, 1998). TOR kinase activity not only controls cell cycle progression through Cln3, but it is also a negative regulator of genes containing stress response elements (STRE's) (Beck & Hall, 1999; Hall *et al*., 1998). The genes encoding trehalose synthase (*TPS1*) and the glycogen synthases (*GSY1*, *GSY2*) are under control of STRE elements (Moskvina *et al*., 1998; Winderickx *et al*., 1996). Therefore, high TOR kinase activity results in a short G_1 phase without reserve carbohydrate accumulation, whereas decreasing TOR activity results in an increase in the G_1 phase duration and carbohydrate accumulation. The results described in chapter 3 show indeed that wild-type cells accumulate high amounts of trehalose and glycogen at low growth rates and low amounts of carbohydrates at high growth rates. In agreement with this, inhibition of the TOR pathway results in physiological changes characteristic of starved cells, including the accumulation of glycogen and an arrest in the G_1 phase (Barbet *et al.*, 1996). Therefore, the TOR pathway seems to be one of the main coordinators of the integration of carbohydrate accumulation in response to the growth rate (figure 1).

Although the TOR pathway may be an important regulator of carbohydrate accumulation, the trehalose and glycogen do not show the same accumulation pattern during growth in continuous cultures (chapter 3). Whereas glycogen accumulation gradually decreases at increasing growth rate, trehalose is strictly only accumulated at low growth rates. Trehalose levels are strongly dependent on the duration of the G_1 phase and it is accumulated at a G_1 phase duration of 5 hours and more. Other variables during growth, like the glucose flux and external sugar concentration, do not have an effect on the amount of accumulated trehalose. Therefore, it seems that the duration of the G_1 phase is the sole determinant in triggering trehalose accumulation. On the other hand, glycogen accumulation seems to be controlled by more than one regulatory pathway. Although an increased growth rate correlates to a decrease in the amount of

accumulated glycogen, at high growth rates other growth conditions seem to influence the glycogen level. When wild-type cells are grown at a constant growth rate of D=0.19 h^{-1}, the glycogen level drops as the glucose flux and the external glucose concentration are increased. Therefore, the glucose flux and concentration may also influence the amount of glycogen. This indicates that a pathway different from the growth rate may regulate the glycogen level at higher growth rates. A good candidate is the cAMP/PKA pathway, which is upregulated by increases in external glucose (Engelberg *et al*., 1990). In agreement with a function of the cAMP/PKA pathway in glycogen accumulation, the activity of glycogen synthase is down-regulated by phosphorylation by a cAMP-dependent protein kinase (Hardy *et al*., 1994). Another pathway that may be involved in regulating glycogen synthase is the CDK Pho85. The Pho85 protein kinase functions independent of the cAMP/PKA pathway and can phosphorylate glycogen synthase in vitro (Huang *et al*., 1998). Furthermore, deletion of Pho85 results in hyperaccumulation of glycogen, indicating direct involvement of Pho85 in glycogen accumulation (Timblin *et al*., 1996). Interestingly, overexpression of *CLN3* resulted in a strong decrease in glycogen accumulation in continuous cultures (chapter 3). Cln3 can activate SBF-mediated transcription at the end of the G_1 phase of, among others, Pho85-activating cyclins *PCL1* and *PCL2* (Baroni *et al*., 1994; Vemu & Reichel, 1995). Therefore, the Pho85 protein kinase may be involved in deactivating glycogen synthase as a downstream response to the level of Cln3 (figure 1).

In nitrogen-limited continuous cultures, the growth rate determines the amount of accumulated trehalose and glycogen (chapter 3). However, in galactose-limited fed-batch cultures the accumulation of carbohydrates itself determines the G_1 length, as shown by the carbohydrate-accumulation deficient mutant (chapter 4). How can the duration of the cell cycle regulate carbohydrate accumulation, whereas carbohydrate accumulation determines the cell cycle duration? This can be explained by the different nutrient limitations that were used in the experiments. In the continuous cultures, the nitrogen amount was limiting for the growth rate, whereas the carbon flux could still regulate other cellular processes like carbohydrate accumulation. In the fed-batch cultures, the only regulating nutrient was the carbon source. Under these growth conditions the amount of available carbon regulates both the growth rate and carbohydrate accumulation. Therefore, carbohydrate accumulation regulates the cell cycle duration only under carbon limitation. Under other growth conditions, the cell cycle duration regulates the accumulation of trehalose and glycogen (chapter 3).

Surprisingly, cells deleted for trehalose and glycogen synthase were able to progress through cell cycle faster than wild-type cells at low consumption rates in galactose-limited fed-batch cultures. The sugar flux and external sugar concentration

were the same during the growth of both strains and are therefore not the main determinant for the duration of the cell cycle under carbon limitation. In the wild type, part of the available galactose is used for the accumulation of carbohydrates, whereas the deletion mutant can use all available sugar for cell cycle progression. Interestingly, wild-type cells degrade part of the reserve carbohydrates at the end of the G_1 phase and thus increase their ATP flux before entry of the S phase. Furthermore, the deletion strain was shown to have a higher ATP flux throughout the growth at low growth rates (Chapter 4; Sillje *et al*., 1999). Therefore, the ATP flux may be involved in triggering cell cycle progression under these growth conditions. As described above, no phosphorylation of the SBF complex was detected in cells with a long G_1 phase. This indicates that the SBF complex is not activated by an increase in the ATP flux generated from carbohydrate degradation. Therefore, another pathway may control cell cycle progression in response to higher ATP fluxes, like the cAMP/PKA pathway. Activation of the cAMP/PKA pathway in G_1 arrested cells results in an increase in *CLN1* and *CLN2* expression, independent of synthesis of new proteins (Hubler *et al.*, 1993; Timblin *et al.*, 1996; figure 1). However, the pathway by which the cAMP/PKA pathway may activate late-G_1 transcription of cells with an elongated G_1 phase duration has yet to be found. Further research may uncover the mechanism by which the different regulatory pathways control cell cycle progression in response to the nutrients in the environment.

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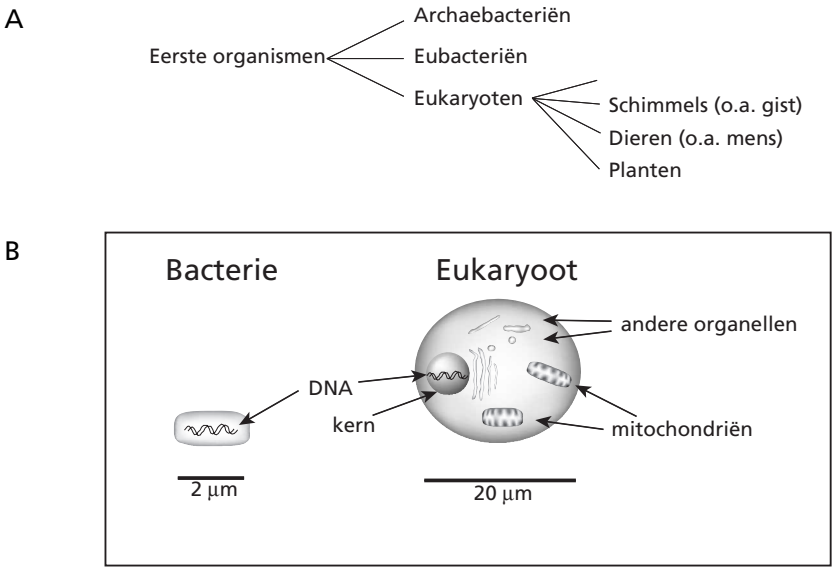
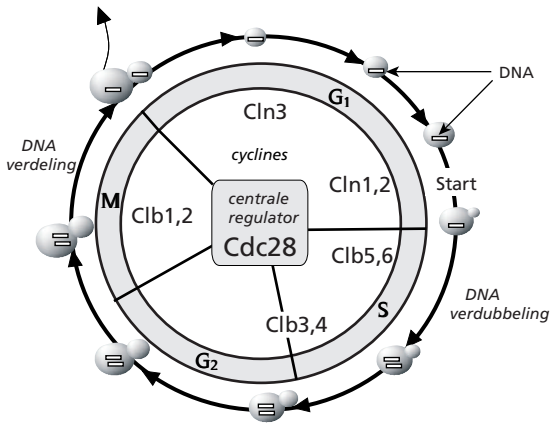
Samenvatting

De gist *Saccharomyces cerevisiae* is bij de meeste mensen bekend als 'bakkersgist' en wordt behalve voor het bereiden van brood onder andere ook gebruikt voor de productie van alcohol in bier en wijn. Net als vele huisdieren, is ook de bakkersgist gedomesticeerd en heeft hij zijn leefgebied bijna uitsluitend in de direkte omgeving van de mens. De mens en gist zijn beide eukaryote organismen, waardoor gistcellen vaak worden gebruikt als een modelsysteem om menselijke cellen te bestuderen. Eukaryote cellen worden gekenmerkt door de verschillende compartimenten waaruit hun cellen zijn opgebouwd (figuur 1). Deze compartimenten worden organellen genoemd en voeren elk een specifiek functie uit, zoals de kern waarin het erfelijk materiaal (DNA) in is opgeslagen en de mitochondriën die als energiefabriek voor de cel functioneren. De andere grote groep van organismen zijn de prokaryoten, oftewel bacteriën, waarvan de meeste veel kleiner zijn dan de eukaryoten en geen organellen bezitten.



Figuur 1: **De afkomst en de verschillen tussen de verschillende organismen.** (A) Organismen hebben zich ontwikkeld in drie soorten: de archaebacteriën, de eubacteriën en de eukaryoten. De eukaryoten hebben zich ontwikkeld tot onder andere schimmels (zoals gist), dieren en planten. (B) De cellen van de eukaryoten en bacteriën verschillen sterk in de opbouw, doordat eukaryote cellen uit verschillende compartementen (organellen) zijn opgebouwd (zoals de kern en de mitochodriën).

Eén van de grootste verschillen tussen gist en de mens is dat gist uit slechts één cel bestaat en de mens uit miljarden. De gistcel kan zich dan ook veel sneller verdubbelen dan de mens: gist kan in anderhalf uur een nakomeling op de wereld zetten, ten opzichte van 9 maanden voor de mens. Gist kan zich zowel op een sexuele manier als op een a-sexuele manier voortplanten (zie ook Hoofdstuk 2, figuur 2). In het eerste geval versmelten cellen van een verschillend 'geslacht' (a-type en α-type) zich, om zo een cel te vormen die beide typen in zich heeft (diploïde cel). Op de a-sexuele manier van reproduceren, kan de cel die gaat delen (moedercel) een identieke nieuwe cel vormen (dochtercel) zonder dat daar andere cellen bij betrokken zijn (je zou het kunnen zien als het zichzelf clonen van een gistcel). De a-sexuele manier van voortplanting is de meest voorkomende manier van reproduktie en deze manier van celdeling wordt beschreven in dit proefschrift.



Figuur 2: **De celcyclus van de bakkersgist.** Om nakomelingen te maken, doorloopt de gistcel de celcyclus. Deze is op te delen in 4 fasen, te weten: de G_1 fase (waarin de cel beslist of hij zal gaan delen), de S fase (waarin het erfelijk materiaal [DNA] wordt verdubbeld), de G_2 fase (waarin de cel zich voorbereid op de uiteindelijke deling) en de M fase (waarin het erfelijk materiaal over de beide cellen wordt verdeeld en de nieuw gevormde cel zich afsplitst). Het gecontroleerd doorlopen van deze fases wordt geregeld door het eiwit Cdc28. Dit eiwit kan actief gemaakt worden doordat cyclines (eiwitten die op een specifiek moment in de celcyclus worden aangemaakt) op het juiste moment binden aan Cdc28, waardoor de cel stap voor stap alle fases kan doorlopen.

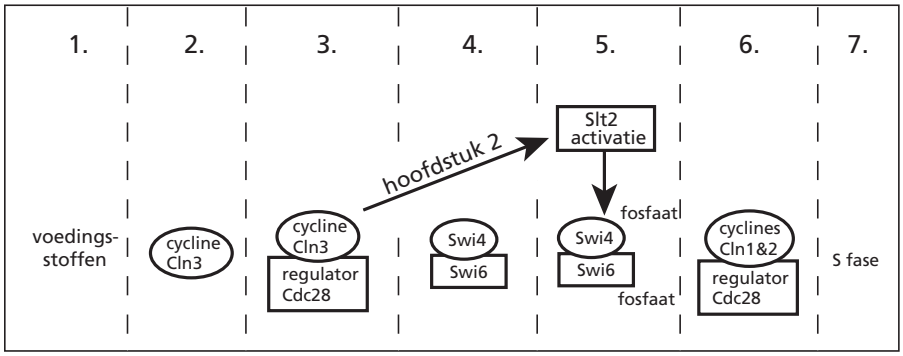
Het mechanisme van reproduktie van de cel (de celcyclus) wordt strak geregeld via achter elkaar verlopende gebeurtenissen. Deze processen die tot verdubbeling van de cel leiden, kunnen worden verdeeld in 4 fasen (zie figuur 2): de G_1 fase (waarin de cel de (mogelijke) deling voorbereid), de S fase (waarin het erfelijk materiaal wordt

verdubbeld), de G_2 fase (waarin de cel de verdeling van materialen over de nieuwe en oude cel voorbereid) en de M fase (waarin het celmateriaal wordt verdeeld over beide cellen en de nieuwe cel wordt afgesplitst). Om deze complexe gebeurtenissen goed te laten verlopen, hebben eukaryote cellen controlepunten ingebouwd op essentiële punten van de celcyclus. Zo is er bijvoorbeeld een controlepunt aan het einde van de S fase die controleerd of het DNA goed is verdubbeld en is er aan het einde van de G_1 fase een punt waarop de cel besluit of hij al dan niet gaat beginnen met de celdeling. Fouten in deze controlepunten kunnen leiden tot ernstige verstoring van de normale celgroei, zoals het ontstaan van ongeremde celdeling door een defect in het controlepunt in de G_1 fase (bij de mens ook wel bekend als kanker). In gist heet dit G_1-fase controlepunt 'Start' en passage van dit punt wordt vooral bepaald door de hoeveelheid voedingsstoffen (nutriënten) in de directe omgeving van de cel.

De manier waarop nutriënten ingrijpen op de celcyclus verloopt via een complex mechanisme van chemische reacties. Deze reacties worden gecatalyseerd door eiwitten (enzymen), die op specifieke momenten in de cyclus kunnen worden aangemaakt en geactiveerd. Het centrale eiwit dat het doorlopen van de celcyclus van de gist *S.cerevisiae* activeerd is Cdc28. Het Cdc28 eiwit kan worden geactiveerd door binding van een cycline; een eiwit dat op een specifiek punt in de celcyclus kan worden aangemaakt (met als uitgezondering de cycline Cln3). De cyclines worden verdeeld in 2 groepen: de G_1-fase specifieke cyclines (Cln's) en de B-type cyclines (Clb's), welke op andere punten tijdens de overige celcyclus-fases worden aangemaakt (figuur 2). In het begin van de G_1 fase wordt Cdc28 geactiveerd door de cycline Cln3, terwijl aan het einde van de G_1 fase de cyclines Cln1 en Cln2 worden aangemaakt, waardoor de cel de S fase in gaat. Door toevoeging van voedingsstoffen aan gistcellen, wordt de hoeveelheid van Cln3 in de cel verhoogd en doorloopt de cel de G_1 fase sneller. Dit gebeurd doordat de activiteit van Cln3-gebonden Cdc28 zorgt voor de aanmaak van het eiwit Swi4. Het Swi4 eiwit bindt aan het eiwit Swi6 en kan zorgen voor de aanmaak van de cyclines Cln1 en Cln2 (figuur 3). Hiervoor moet het complex van Swi4 en Swi6 (het SBF-complex) nog wel eerst geactiveerd worden door het aanbrengen van een fosfaat groep. Deze activatie door fosforylatie wordt uitgevoerd door het eiwit Slt2, dat deel uitmaakt van de PKC signaleringsroute. Tot op heden was het onduidelijk hoe het eiwit Slt2 wordt geactiveerd aan het einde van de G_1 fase.

In hoofdstuk 2 staat beschreven dat de lengte van de celcyclus bepaald wordt door de snelheid waarmee de cel suiker gebruikt (de suiker flux), welke bepalend is voor de hoeveelheid gefosforyleerd Slt2. De duur van de G_1 fase kan worden verlengd door de snelheid waarmee de suiker galactose aan gistcellen wordt toegevoegd te verlagen. Als gevolg van deze lagere suiker-consumptie snelheid wordt het eiwit Slt2

minder gefosforyleerd. Om te kijken of de hoeveelheid gefosforyleerd Slt2 ook inderdaad bepalend is voor de duur van de G_1 fase, zijn verschillende technieken gebruikt. Door het verwijderen van het Slt2-eiwit uit de gist (*slt2* deletie) en door de hoeveelheid Slt2 in de cel te verhogen (*SLT2* overexpressie) is de hoeveelheid van gefosforyleerd Slt2 beïnvloed. De duur van de G_1 fase bleek hierbij afhankelijk te zijn van de hoeveelheid gefosforyleerd Slt2; hoe meer gefosforyleerd Slt2, hoe korter de G_1 fase duur. Ook door verhoging van de hoeveelheid van G_1-fase cycline Cln3 in de cel (*CLN3* overexpressie) werd de hoeveelheid gefosforyleerd Slt2 sterk verhoogd en de G_1-fase duur drastisch verkort. Hierdoor lijkt de hoeveelheid Cln3 bepalend te zijn voor de hoeveelheid gefosforyleerd Slt2 in de cel en dus voor de duur van de G_1 fase van de celcyclus (figuur 3).



Figuur 3: **Stapsgewijze regulatie van de G_1 fase van de celcyclus.** Door de aanwezigheid van voedingsstoffen (1) wordt in de gistcel de hoeveelheid van het eiwit Cln3 verhoogt (2). Hierdoor kan deze cycline binden aan de regulator Cdc28, zodat deze actief wordt (3) en zorgt voor de productie van het eiwit Swi4. Swi4 kan binden aan het eiwit Swi6 (4) en deze kunnen samen actief worden gemaakt door het aanbrengen van een fosfaatgroep door het eiwit Slt2 (5). Nadat het Swi4/Swi6-koppel actief wordt, worden de eiwitten Cln1 en Cln2 aangemaakt. Deze kunnen binden aan de centrale regulator Cdc28 (6), waardoor deze actief wordt en zorgt voor het opstarten van de S fase (7). In hoofdstuk 2 wordt beschreven dat de hoeveelheid van de cycline Cln3 zorgt voor een hogere activiteit van het eiwit Slt2, waardoor de G_1 fase sneller kan verlopen.

Onder omstandigheden dat zeer weinig suiker aan de gistcel wordt gegeven doet de cel er lang over om door de G_1 fase te komen. De cel gaat zich dan voorbereiden op een periode waarin hij geen suiker meer zal hebben, waarin hij zal moeten overleven. Om te overleven kan de cel suiker opslaan in de vorm van trehalose (2 suikermoleculen aan elkaar) en glycogeen (een lange keten van suikermoleculen).

Maar waardoor besluit de cel nu om trehalose en glycogeen op te bouwen? In hoofdstuk 3 is hier een studie naar gedaan en is gevonden dat de beslissing om trehalose en glycogeen op te bouwen wordt bepaald door de groeisnelheid van de cel. Eerdere studies hebben al laten zien dat op het moment dat de cel een tekort heeft aan nutriënten, zowel trehalose als glycogeen worden opgebouwd en dat dit samenvalt met een lager suikergebruik, grote veranderingen in de hoeveelheid tussenprodukten van suiker-afbraak in de cel en een vermindering in de groeisnelheid. Door het opleggen van specifieke groeisnelheden aan de gistcel (met behulp van een 'continu culture', waarin de groeisnelheid constant kan worden gehouden) is gebleken dat de groeisnelheid van de cel bepalend is voor de hoeveelheid trehalose en glycogeen die worden opgebouwd. Verder bleek dat andere factoren, zoals de suikerconcentratie buiten de cel, de hoeveelheid aan suiker-afbraak produkten in de cel en het suikergebruik niet bepalend zijn voor de hoeveelheid opbouwde reserve-suikers. Door de hoeveelheid Cln3 in de cel te vergroten (en zo de G_1-fase duur te verkleinen) is gebleken dat de hoeveelheid trehalose die wordt aangemaakt direkt afhankelijk is van de duur van de G_1 fase. De glycogeen hoeveelheid lijkt ook nog door andere factoren dan de lengte van de G_1 fase te worden bepaald.

In hoofdstuk 4 wordt dieper ingegaan op de functie van trehalose en glycogeen voor de cel. Hier wordt beschreven dat zowel trehalose als glycogeen kunnen dienen als reservestof, om lange periodes zonder suikerbron te overkomen. Verder wordt beschreven dat door de aanwezigheid van beide reservestoffen cellen sneller kunnen beginnen met groeien als reactie op vers toegevoegde suikers. Trehalose en glycogeen worden onder langzame groeisnelheden opgebouwd in de G_1 fase van de celcyclus en weer afgebroken aan het einde van de G_1 fase. Tot nu toe was gedacht dat de hoeveelheid energie die vrij komt door het gebruik van de reserve suikers nodig is voor de cel om te gaan delen. Door het bestuderen van cellen die geen trehalose en glycogeen kunnen opbouwen, is gebleken dat deze reservestoffen niet noodzakelijk zijn om te kunnen groeien onder deze omstandigheden. Het blijkt zelfs dat de opbouw van reservestoffen kan leiden tot een vertraging in de celcyclus, doordat cellen die geen reservestoffen kunnen opbouwen sneller hun celcyclus kunnen voltooien wanneer ze dezelfde hoeveelheid suiker tot hun beschikking hebben. Hoewel trehalose en glycogeen dus beide een positieve werking hebben op de overleving van gistcellen en op de snelheid waarmee deze op de omgeving kunnen reageren, hebben ze ook een negatief effect op het doorlopen van de celcyclus. Gedurende de evolutie heeft de cel echter voortdurend voor de keuze gestaan om óf zich voor te bereiden op overleving óf zo snel mogelijk nieuwe nakomelingen te produceren. Logischerwijs kiest de gistcel allereerst voor het hamsteren van voedsel voor de overleving, voordat hij zijn

nakomelingen op de wereld zet. Andere eukaryote organismen bouwen veelal eenzelfde zekerheid in voor de toekomst van zijn soort, zoals de economische zekerheid die de mens inbouwd voor zij kinderen op de wereld zet. Misschien zou de bakkersgist daarom ook als een goed modelsysteem voor de mens te gebruiken zijn in de 'grotere vraagstukken' van het menselijk leven.

Dankwoord

"Nou, gewoon bedankt!"

Al zoveel woorden gebruikt in de laatste jaren, die mensen dan wel gelukkig maakten of tot wanhoop dreven (de hoeveelheid dan vooral). Aangezien iedereen toch wel aan zou moeten voelen in welke mate ze hebben bijgedragen aan het tot stand komen van dit proefschrift, weegt de meerwaarde van het opnoemen van al deze namen volgens mij niet op tegen het 'leed' dat geleden zou kunnen worden door het vergeten te noemen van sommige personen. Naast de wetenschappelijke bijdragen van de mensen welke overduidelijk in dit boekje te zien zijn, wil ik hier vooral ook al diegene bedanken die me tussen de proeven door en buiten de werktijd om hebben vermaakt. Zonder jullie motiverende werking was dit proefschift nooit tot stand gekomen.

Lieve mensen, bedankt!!!

List of Publications

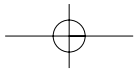
J.J.P. Baars, H.J.M. Op den Camp, J.W.G. Paalman, C. van der Drift, G.D. Vogels, L.J.L.D. Van Griensven, V. Mikes (1995). Purification and Characterisation of Glutamine Synthetase from the Commercial Mushroom Agaricus bisporus. *Current Microbiology* **35**: 108-113.

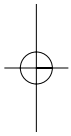
H.H. Sillje, J.W.G. Paalman, E.G. ter Schure, S.Q. Olsthoorn, A.J. Verkleij, J. Boonstra, C.T. Verrips (1999). Function of trehalose and glycogen in cell cycle progression and cell viability in Saccharomyces cerevisiae. *J Bacteriol.* **181**(2):396-400.

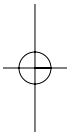
J.W.G. Paalman, S.H. Slofstra, A.J. Verkleij, J. Boonstra, C.T. Verrips. Trehalose and glycogen accumulation is dependent on the growth rate in *Saccharomyces cerevisiae*. *submitted for publication*.

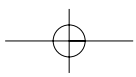
J.W.G. Paalman, A.van Walré de Bordes, A.J. Verkleij, J. Boonstra, C.T. Verrips. Phosphorylation of the MAP kinase Slt2 induces cell cycle progression in *Saccharomyces cerevisiae*. *submitted for publication*.

R. Verwaal, J.W.G. Paalman, A. Hogenkamp, A.J. Verkleij, C.T. Verrips, J. Boonstra. Hxt5 is expressed upon conditions that induce low growth rates in *Saccharomyces cerevisiae*. *manuscript in preparation*.









Curriculum Vitae

Hans Paalman werd op 31 augustus 1970 te Deventer geboren. Zijn middelbare schoolopleiding heeft hij gevolgd aan het Titus Brandsma Lyceum te Oss, alwaar hij in 1988 het diploma Atheneum heeft behaald. In hetzelfde jaar begon hij de studie Scheikunde aan de Katholieke Universiteit Nijmegen, welke hij in 1996 succesvol afronde. Tijdens deze studie heeft hij zich gespecialiseerd in de richtingen Microbiologie (Prof. Dr. G.D. Vogels) en Moleculaire Celbiologie (Prof. Dr. W.M. de Vos, NIZO). In oktober 1996 is hij begonnen als Assistent in Opleiding aan de Universiteit Utrecht bij de vakgroep Moleculaire Celbiologie (Prof. Dr. A.J. Verkleij en Prof. Dr. Ir. C.T. Verrips) onder begeleiding van Prof. Dr. J. Boonstra. Tijdens deze periode werd het in dit proefschrift beschreven onderzoek verricht.